The Behaviour and Chemistry of Recruitment and Alarm in Social Insects

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Declaration

I hereby declare that this thesis has not been, and will not be, submitted in whole or in part to another University for the award of any other degree.

Summary

This thesis initially focuses on the chemical ecology of two species of ant which are common in the United Kingdom; *Lasius flavus*, the yellow meadow ant and *Lasius niger*, the common black ant. The first data chapter explores the constituent chemicals present in 3 major exocrine glands located in the gaster of *L. flavus* and discusses their potential functions. The work presented here also highlights the need for the comparative study of the chemical composition of glands. The second data chapter investigates how chemicals present in hindgut extractions of *Lasius niger* vary with the temporal caste they belong to (nurse or forager), and subsequently looks at how those chemicals may be suited to the tasks performed by that caste. The third data chapter describes the development of a highly sensitive methodology to identify low-concentration pheromones that uses a combination of analytical chemistry and behavioural bioassays. This methodology was used to identify two attractive pheromones of *L. flavus*, one is a trail pheromone used during foraging while the other is an alarm pheromone used to warn nest-mates of danger. The trail pheromone is the lowest concentration pheromone to be successfully identified in ants to date. This chapter also highlights the need to perform comparative behavioural bioassays to demonstrate the true function of putative pheromones. The final data chapter then investigates the source of alarm signals in *Nasutitermes corniger* termites and assesses the differential responses of workers and soldiers. This chapter then goes on to elucidate the colony-level effects of alarm on the regularity of repairs made to experimentally manipulated foraging galleries.

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1 Introduction

1.1 Eusocial Insects

1.1.1 Eusociality

In a literal sense the word eusocial means 'truly social'. For a group of animals to be defined as eusocial, they must fit the following criteria: the cooperative care of brood, overlapping generations within a single colony of adults, and a division of labour of non-reproductive groups (Crespi and Yanega, 1995; Wilson and Hölldobler, 2005). When discussing eusocial insects, the groups that often come to mind are the eusocial Hymenoptera: bees, wasps and ants. These are certainly the most widespread and most frequently encountered of the eusocial insects. Following closely behind in notoriety are the termites in the order Blattodea.

1.1.2 Division of labour

The work of eusocial insect colonies is organised by systems that incorporate some form of division of labour (Beshers and Fewell, 2001). At the most primitive level, all individuals in a colony are capable of reproducing and are morphologically identical, but only a subset of individuals will actually reproduce. This is the case in ants of the genus *Dinoponera*, where the alpha female uses chemical signals to exert dominance over her nestmates and thereby monopolises reproduction in the colony (Monnin and Ratnieks, 2001).

In more advanced species, the reproductive division of labour is accompanied by morphologically distinct castes. This is the case in the majority of eusocial insect species, such as the commonly studied honeybee, *Apis mellifera* (Dietemann et al., 2013), and the common wasp, *Vespula vulgaris* (Harris and Beggs, 1995). This is also the case in the vast majority of ant species (Hölldobler and Wilson, 1990) and all species of termite (Bignell et al., 2011). In colonies that utilize this method of organisation, most of the individuals are non-reproductive workers, with a minority of reproductive individuals referred to as queens (Beshers and Fewell, 2001), or kings and queens in the termites (Bignell et al., 2011).

In many species of ant and termite, the non-reproductive workers in a colony may be further separated into multiple morphological castes which perform more specific tasks. For example, many species in the ant genus *Pheidole* possess both major and minor workers (Wilson, 2003). As their name suggests, the major workers are larger than the minors and have much larger heads relative to the rest of their bodies. Their large heads accommodate greatly enlarged muscles which power the mandibles, giving them a powerful bite (Wilson, 2003). This morphological specialization allows the major workers to specialize in their task performance; they act as effective soldiers for the colony and use their strong jaws to mill seeds. However they cannot perform other vital tasks such as nest maintenance or brood care. These non-defensive tasks must be performed by the more dextrous minor workers (Holley et al., 2016). More complex worker polymorphism is found in some species of leafcutting ants. Many ants of the genus *Atta* possess at least four different castes of non-reproductive worker (Hölldobler and Wilson, 1990). The smallest, known as minims, are small enough to

pluck single hyphae from the fungus which the ants grow for nourishment. The largest, known as supermajors, have massive jaw muscles and a powerful bite which they use to deter invaders (Hölldobler and Wilson, 2010). The most extreme worker size polymorphism is found in the ant species *Pheidologeton diversus* where the largest workers have a dry weight approximately 500 times heavier than the smallest (Moffett, 1987).

A soldier caste of workers is found in almost all species of termite, and they are perhaps specialised even more than those of the ants (Bignell et al., 2011). Termite soldiers possess a huge variety of specialised shapes and functions. *Termes panamensis* soldiers possess asymmetrically curved mandibles which can be pressed together in a way that deforms the mandible shape, storing energy. This energy can be released in a rapid mandible strike that is powerful enough to decapitate invading ants (Seid et al., 2008). Soldiers of the genus *Nasutitermes* do not possess mandibles at all, instead they have a nozzle-like rostrum from which an adhesive, poisonous and corrosive secretion can be fired to repel invaders (Prestwich, 1979a).

A further level of workload organisation is frequently observed in eusocial insects known as temporal polyethism, and can be present regardless with or without worker polymorphism (Robinson, 1992). Where this system exists, workers perform different tasks based on their age. In general young workers perform tasks that can be completed without leaving the nest and are therefore relatively safe, such as grooming the queen and tending to larvae and pupae. Older workers on the other hand perform the riskier tasks such as foraging and defending the nest from invaders (Robinson, 1992); concomitantly they also have a tendency to be more aggressive (Seid and

Traniello, 2005; Shorter and Tibbetts, 2009). These tasks often result in workers leaving the nest and are therefore associated with a higher rate of mortality due to predation or environmental hazards. This system ensures that it is most likely that a worker performs a lifetime of work before perishing, and the colony therefore benefits from the maximum possible output of the workforce. This system has been observed in wasps, bees, ants and termites (Depickère et al., 2004; Hinze and Leuthold, 1999; Johnson, 2008; Naug and Gadagkar, 1998).

1.2 Chemical Signalling in Social Insects

When observing a colony of eusocial insects, their collective movements can appear so coordinated that it is easy to assume there is a single organising entity. This observed coordination stems from the emergent properties of simple interactions between individuals (Bonabeau et al., 1997). These interactions are very frequently mediated by chemical communication in the form of semiochemicals (Vander Meer et al., 1998).

Semiochemicals can be divided into three broad classes: allomones, kairomones and pheromones. The term allomone refers to a chemical released by an individual of one species that affects a member of another species, but only the originator of the chemical receives benefit. One example is an allomone produced by the larvae of the Neuroptera (lacewing) species *Lomamyia latipennis*. The larvae live within *Reticulitermes* termite nests and feed upon adult termites. They subdue the termites using an aggressive allomone secreted from their abdomen which paralyses termites, ultimately leading to their death. This allomone is so potent that a third instar *L. latipennis* larvae was observed to incapacitate 6 adult termites simultaneously

(Johnson and Hagen, 1981). Kairomones are chemicals emitted by one species which are detected by another species to the benefit of the receiver. An example is the volatile organic compounds (VOCs) used by the aphid *Aphis fabae* to locate its host plant *Vicia faba* (Webster et al., 2008). Pheromones are chemicals emitted by one species that influence the behaviour of other members of the same species. The use of pheromones to communicate is widespread throughout almost all forms of organism, but the eusocial insects perhaps display the most variety in their use (Vander Meer et al., 1998). Some examples of pheromone functions are: recognition pheromones that allow individuals to recognise nestmates (Kather and Martin, 2015), fertility signalling pheromones which are sometimes used by reproductive individuals to subdue reproductive behaviour in workers (Oystaeyen et al., 2014), repellent pheromones to warn conspecifics to avoid an area (Robinson and Jackson, 2005) and recruitment pheromones which attract conspecifics to an area (Dussutour et al., 2009). The two major uses of recruitment pheromones in the eusocial insects are as alarm pheromones and trail pheromones.

1.2.1 Trail pheromones

The term trail pheromone describes a chemical that is laid on the ground which elicits attraction and following responses in conspecifics. As the function of these pheromones relies on them being deposited on a solid surface, their use is widespread among the terrestrial eusocial insects such as ants and termites, but they have also been discovered in some species of stingless bee (Jarau et al., 2006). They are used by many ant and termite species to aid in the organisation of foraging effort. In the simplest form of organisation a single trail pheromone is used. When a scout ant

locates a resource in the environment (this can be anything the colony requires, such as water, displaced brood but most often food) of a quantity that they cannot retrieve in one trip, they lay a trail of an attractive chemical back to the nest. This allows subsequent foraging ants to navigate quickly to the resource, and if there is still an excess when they have gathered their share they will 'top up' the trail by laying more of the same chemical. Once the resource is fully exploited, ants will stop laying a trail and the chemical will evaporate. This system allows ant colonies to quickly exploit resources in the surrounding environment (Morgan, 2009). A combination of negative feedback due to crowding at a resource, along with evaporation of the trail pheromone prevents ants from becoming 'locked in' to less rewarding foraging sites (Czaczkes et al., 2013; Grüter et al., 2012).

To produce and secrete pheromones, ants possess an array of exocrine glands which each produce a variety of chemicals (Hölldobler and Wilson, 1990). The trail pheromones of ants are invariably laid using the gaster (Morgan, 2009), which refers of the rearmost section of the abdomen. There are three major exocrine glands present in the gaster of all ants; the Dufour gland, the poison gland and the hindgut. To identify trail pheromones, one should therefore investigate these glands. The morphology of the three glands can vary quite drastically between species, but

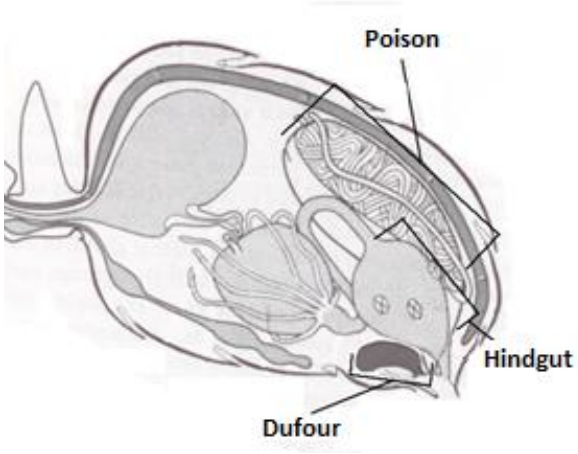

Fig. 1 – A cross section of a gaster from a Formicine ant. Three major exocrine glands are labelled. The diagram is modified from Hölldobler and Wilson (1990).

a cross section of a Formicine gaster is shown in Figure 1 as a general example. The subject of ant trail pheromones has been reviewed in great detail by Morgan (2009), and not a great deal of progress has been made on characterizing trail pheromones since then.

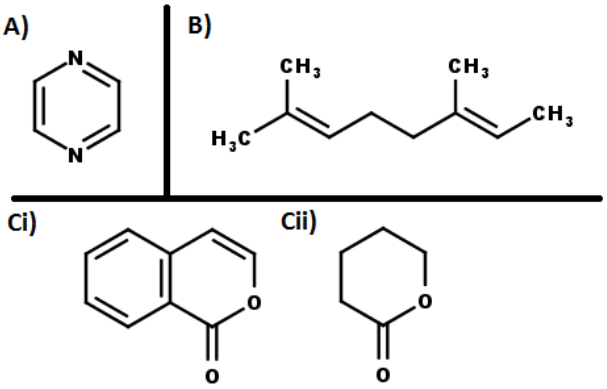

Fig 2. – The basic structures of trail pheromones from different subfamilies and glandular sources in ants. A) and B) are both found frequently in Myrmicine ants. A) Pyrazines are usually produced in the poison gland, while B) terpenes are produced in the Dufour gland. C) are trail pheromones found in the hindguts of Formicine ants. Ci) is a dihydroisocoumarin while Cii) is a δ-lactone.

The gland where trail pheromone is produced and stored varies between the different subfamilies of ants, as does the general chemical structure of the pheromones. The trail pheromones of Myrmicine ants tend to be produced in the poison gland and are usually laid from the tip of the sting. The chemical structures are usually pyrazines; a five or six-membered carbon ring which incorporates two nitrogen atoms. Diagrams of the basic structure of pyrazine trail pheromones, and others from different subfamilies discussed here are shown in Figure 2. The communication systems of the Myrmicine ants appear to be simpler to those of the more derived Formicines, as the pyrazines appear to act as a broad spectrum attractant and poison. They are used by Myrmicine ants as a toxin, alarm pheromone and trail pheromone. There are exceptions to this rule; the species *Aphenogaster albisetosus* is reported to use a mixture of (*S*)- and (*R*)-4-methyl-3-heptanone as its trail pheromone (Hölldobler et al., 1995). This is surprising, because the same compound has been identified as the

alarm pheromone of many other myrmicine species and is usually located in the mandibular gland in the head of the ant (Blum et al., 1968; McGurk et al., 1966; Moser et al., 1968; Norman et al., 2017). Some other species of Myrmicine ants, such as *Monomorium pharaohnis* and *Solenopsis invicta,* produce and store their trail pheromone in the Dufour gland. Trail pheromones identified from this gland are usually sesquiterpenes.

The trail pheromones of Formicine ants are found exclusively in the hindgut. Discovery of these pheromones came somewhat later than those of other glands, probably due to the relatively low concentrations of pheromone present. While the pheromones described above from other gland types were present in nanogram concentrations per gland, formicine trail pheromones were present at only picogram concentrations. The structure of formicine trail pheromones is always based around one of two structures, either an isocoumarin or a δ-lactone. Usually multiple variations of the trail pheromone compound are present, but only one or two may elicit activity in trail following bioassays (Morgan, 2009). Presumably this variation allows for evolutionary selection of pheromone chemical, but this has not been investigated. So far the trail pheromones of Formicine ants in the tribe Lasiini have received relatively little attention, despite comprising some of the most numerous species in Europe (Czechowski et al., 2013a). Just two species have had their trail pheromones identified thus

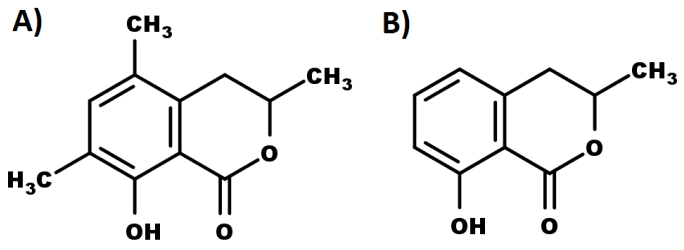

Fig. 3 – The only two trail pheromones identified from *Lasius* ants. A) 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin was discovered in *L. niger* hindguts. B) Mellein was discovered in *L. fuliginosus* hindguts.

far, *Lasius niger* and *Lasius fuliginosus.* The trail pheromone of *L. niger* was found to be 3,4-dihydro-8-hydroxy-trimethylisocoumarin (Bestmann et al., 1992), while *L. fuliginosus* was found to use 3,4-dihydro-8-hydroxy-3-methylisocoumarin (Kern et al., 1997), commonly known as mellein. The structures of these two pheromones are shown in Figure 3. The trail pheromone of the yellow meadow ant, *Lasius flavus,* is yet to be elucidated. To date this species has been almost entirely neglected by the chemical ecology literature, despite its population outstripping that of the more commonly studied *L. niger* in many habitats (Czechowski et al. 2013). *Lasius flavus* is an important ecosystem engineer in grassland ecology (Boots & Clipson 2013; Vlasáková & Raabová 2009), and *L.flavus* workers seem to place more reliance on the social information provided by pheromone trails than does *L. niger* (Jones, 2014). The genus *Lasius* also contains a major pest species, *Lasius neglectus* (Seifert, 2000). Trail pheromones have been shown to increase the efficiency of poisoned baits (Tatagiba-Araujo et al., 2012), so further work on the trail pheromones of species in the genus *Lasius* may produce improved methods of selectively controlling this pest.

1.2.2 Alarm pheromones

Eusocial insects use alarm pheromones to warn their nestmates of nearby threats and the response of nestmates to the alarm signal can vary depending on the species. In ants, the most frequently observed response is attraction to the source of the alarm and a heightened tendency to aggress. A more erratic pattern of running and a higher run speed can also be observed (Hölldobler and Wilson, 1990). Alarm pheromones differ to trail pheromones in that they are very frequently multicomponent and in

some species are emitted from multiple glands simultaneously (Ayre and Blum, 1971; Witte et al., 2007b).

The mandibular gland situated in the head of the ant is always the source of at least one component of the alarm signal (Hölldobler and Wilson, 1990), and sometimes chemicals from the poison gland and Dufour gland are also important in eliciting the full response (Ayre and Blum, 1971; Witte et al., 2007b). The chemical structures of alarm pheromones in the mandibular gland varies between species, but overall there is less diversity than in trail pheromones. In almost all species the chemicals released from the mandibular gland are volatile ketones, aldehydes or sometimes alcohols (Crewe et al., 1972; Crewe and Blum, 1970; Hölldobler and Wilson, 1990; Norman et al., 2017).

The chemicals produced in the poison gland are more variable between the different subfamilies of ants. For example the Myrmicinae use their sting to inject venom from their poison gland and pyrazines are often present in this secretion. As discussed above, these pyrazines are attractive to workers as they are often used as trail pheromones. It is not unreasonable to deduce that they may act in concert with mandibular compounds in eliciting a full alarm response, because venom is released when Myrmicine ants sting invaders, although this has not been investigated. The Formicinae do not possess a sting and instead produce large amounts of formic and acetic acid in their poison glands which they spray on invaders (Attygalle and Morgan, 1984; Abraham Hefetz and Blum, 1978; Wilson and Regnier, 1971). The corrosive action of formic acid is thought to be enhanced by large amounts of undecane which is used as a wetting agent and is frequently found in the Dufour gland of Formicine ants

(Lenz et al., 2012). The contents of the mandibular gland, Dufour gland and poison gland, both in isolation and in combination, have all been found to elicit alarm-like behaviour in many species of Formicine ant (Hölldobler and Wilson, 1990; Piek, 2013). The specific function of each gland with regard to eliciting alarm response behaviour has yet to be investigated.

1.3 Analytical Techniques in Chemical Ecology

The first identified pheromone was bombykol, which is the sex pheromone of the silkworm moth, *Bombyx mori* (Butenandt et al., 1959). 3.4g of bombykol was extracted and isolated from a staggering 500,000 'fragrance glands' and was identified using a combination of infra-red (IR) and ultra-violet (UV) spectroscopy. The first discovered trail pheromone was that of a termite, *Reticulotermes virginicus* (Matsumura et al., 1968). The researchers started by macerating 385g of termites in a solvent, then performed column chromatography, thin-layer chromatography and 2 separate stages of gas-liquid chromatography to produce approximately 1µg of pure trail pheromone. A combination of mass spectrometry, nuclear magnetic resonance (NMR) and UV and IR spectroscopy revealed the structure to be 3,6,8-dodecatriene-1-ol. It was noted that termites followed a trail of this substance, but no behavioural data were provided. Tumlinson et al. (1971) later discovered the first ant-trail pheromone, that of the leafcutting ant *Atta texana*. As with the two studies mentioned above, a large amount of biological material was required for this to be achieved. In total, 3.7kg of ants were used in the extraction of the compound. Vapour phase chromatography was used to purify the substance, and four separate gas-liquid

chromatography stages were used. A combination of NMR, mass spectrometry and infra-red spectroscopy indicated that the structure was 4-methylpyrrole-2-carboxylate. Trail following tests were vaguely described, but again no behavioural data were provided and no strict comparisons were made with natural trail following behaviour.

These early identifications required massive amounts of biological material in order to isolate large quantities of pure pheromone which could then be exposed to multiple analytical techniques to assign potential structures. Perhaps the most significant subsequent development which allowed the field of chemical ecology to rapidly expand was the creation of gas chromatographs linked to highly sensitive mass spectrometer (GCMS) instruments that were capable of total ion monitoring (recording a wide range of ionic masses generated by electron ionization of analytes). These new mass spectrometer devices allowed researchers to work with far smaller samples, and advances in gas chromatography allowed more straightforward separation of compounds in complex mixtures. Linking the two techniques, GC and MS, together made it possible to generate spectroscopic data for a large number of analytes in a sample in a single 'run'. The development of capillary columns further refined the ability of researchers to work with small quantities of analytes, as they provide higher separation resolution and reduced column bleed (which may obscure target analytical peaks) when compared with packed columns (Barry and Grob, 2007).

Although these developments allowed the field to grow rapidly, they do have their own experimental limitations. GCMS typically relies upon the injection of liquid samples. This often means that samples must be dissolved in a solvent, which both dilutes analytes thereby making them more difficult to detect, and can introduce

contaminants to a sample if solvents are impure. To try and remedy these issues, a solid sample injection technique was developed (Morgan, 1990). This method involves sealing glands in small glass capilliaries, which are then heated and crushed in the injection inlet of the GC, allowing volatilised compounds to flow onto the column. This method has been successfully used to identify many insect pheromones (Bagnères and Morgan, 1991; Martin and Drijfhout, 2009; Morgan, 2009; Oldham et al., 1994), but does present some challenges. It requires the use of a custom-built crushing device which can hold glass capilliaries in the inlet of the GC, and requires specialised modification of the inlet itself to accommodate the device. It also runs the risk of allowing crushed glass to enter the GC column, and of introducing contaminants from the glass into the column.

Another sensitive technique used in the field is Solid Phase Microextraction (SPME). This method involves using a specialised fibre (usually made of polysiloxanes) to adsorb a sample, this can be used to collect volatile chemicals from the headspace around the target organism (Agelopoulos and Pickett, 1998), or can be brushed onto a surface to sample chemicals via contact (Monnin et al., 1998). This fibre can then be placed into the GCMS inlet and heated, thus releasing the chemicals onto the GC column. This method has the advantage of solvent-free injection, and allows the sampling of volatile chemicals from the air. It has been successfully used to identify pheromones in ants, such as cuticular hydrocarbons (Martin and Drijfhout, 2009) and the trail pheromone of the argentine ant (Choe et al., 2012). A disadvantage, which is also present in the solid sample injection technique described above, is that a chromatogram of even a single gland can contain hundreds of signals. The

behaviourally important compounds are often not the most abundant, and sometimes, even with careful programming of the gas chromatograph, the necessary separation resolution cannot be achieved. This can lead to important peaks of interest being obscured by larger, non-relevant peaks. Even if sufficient separation of all peaks can be achieved via GC, a researcher can face the problem of attempting to identify hundreds of different compounds, before trying to determine which may be putative pheromones. This can be a very time consuming process.

To attempt to solve this issue some researchers have proposed the use of a programmable 'Deans Switch' to divert the flow of carrier gas from the GC column into a recovery vial. This allows the recovery of samples while the GC is running. These samples can be used to test for activity in bioassays, thus providing data on the retention times of compounds that elicit the behaviour of interest. This can considerably reduce the number of peaks that must be identified, but also has the disadvantage of splitting the original sample. This may result in compounds falling below the limit of detection of the MS. The amount of compound recovered from the GC may also be too low to elicit a response in bioassays.

Notwithstanding these technical difficulties, any putative pheromones identified using the above analytical chemistry techniques, must have their functions confirmed by testing authentic standards in behavioural bioassays. The concentrations of compounds tested in bioassays should mimic those found naturally, and the response should be compared with those elicited by extracts taken directly from the organism. The bioassays must be behaviourally relevant to the organism, and must be designed in such a way that the results can be analysed statistically. Often this vital step is

neglected, or behavioural work is performed quickly, almost as an afterthought. Another important consideration is that different pheromones can elicit similar results in a bioassay, even if their primary biological functions are completely different. As discussed above, both alarm and trail pheromones elicit attraction in workers, so a bioassay to test only for attraction would not be able to differentiate an alarm pheromone from a trail pheromone. A frequently used assay to test whether a pheromone stimulates an alarm response is a simple test for attraction, which may misidentify the true function of tested pheromones (Fujiwara-Tsujii et al., 2006; Witte et al., 2007b; Yu et al., 2014).

There are two frequently used bioassays to test whether compounds function as trail pheromones in ants; the T-maze and the circle assay. To perform the T-maze assay, a T-shaped platform is connected to an ant colony using a bridge. A food source is then placed in the centre of the bridge to allow ants to form a natural pheromone trail. The chemicals to be tested can then be laid on either branch of the T, and the number of ants counted which choose either branch. A putative trail pheromone can be tested against a solvent control, and if the number of ants choosing the test trail is significantly higher than 50%, a positive result can be claimed (Morgan, 2009). This assay does pose some problems. The flow of ants up to the branches must be carefully controlled – too few results in a non-meaningful result as not enough ants make the decision. Too many ants can result in overcrowding where ants simply do not have sufficient space to make a true decision. The results can also be highly variable, and there are many potentially confounding factors to be controlled, such as lighting position, air flow position or position of the researcher. Finally, ants must be

immediately removed from the assay once a decision has been made to prevent double counting of individuals. This can be difficult depending on the number of ants participating, and can easily lead to the release of alarm pheromones if an ant is removed clumsily. As discussed above, alarm pheromones frequently cause attraction to nearby ants so this is likely to affect the result. Finally, this assay cannot provide any data on how intense the following response is, as the only measurement is the proportion of ants making the decision to follow a trail. It has been demonstrated that incorrect use of this assay can result in artificially poor trail following response (Czaczkes et al., 2017).

Circle assays have been described whereby a circle is drawn with 1cm radii measured. A pheromone trail is laid around the circle and an ant is placed in the centre. The number of radii the ants cross is then counted before the ant leaves the trail. This assay has advantages over the T-maze; the intensity of the following response is measured and there are less confounding variables. However potential problems arise when deciding when an ant has left the trail, occasionally ants can veer away from a trail before rejoining it, so deciding on when an ant leaves a trail can be subjective. Also, the ant must be placed in the centre of the circle. Invariably ants are alarmed when handled with forceps (Regnier and Wilson, 1968; Wilson, 1958), and alarmed ants may not perform well in a trail following test that focuses on behaviour in a foraging context .

Misidentified trail pheromones have been reported previously. Huwyler et al. (1975) originally described the trail pheromone of *Lasius fuliginosus* as a combination of 6 fatty acids and even used an 'S' shaped trail following assay to confirm this. However

these compounds were later shown to not elicit trail following, and the trail pheromone was re-identified as mellein (Kern et al., 1997). Cavill et al. (1980) identified hexadecanal as an attractive pheromone present in the ventral gland of *Linepithema humile* ants, and researchers subsequently began referring to the compounds as a trail pheromone (Key and Baker, 1982a, 1982b; Van Vorhis Key et al., 1981). This compound was later actually found not to be laid by *L. humile* in its trails, but the compounds dolichodial and iridomyrmecis were instead identified as trail pheromones. The trail pheromone of *Monomorium pharaohnis* was originally misidentified as monomorines I and II, but later corrected to faranal (Morgan, 2009). Careful and robust analytical chemistry combined with rigorous bioassays may have avoided these misidentifications, and the work presented in this thesis aims to pair these methods successfully.

1.4 Aims

As discussed above, there are gaps in the methods currently used in the field of chemical ecology where the challenge is to identify low concentration pheromones from complex mixtures. The major aim of this thesis is to bridge this gap by developing a highly sensitive methodology that uses a combination of analytical chemistry and robust, relevant bioassays. This will be performed using the commonly studied *Lasius niger* and the poorly studied *Lasius flavus* as model organisms. This will also expand the small library of chemicals currently known to be produced by ants in the *Lasius* genus. The methods will also be used to explore whether glandular chemicals are related to temporal caste in systems where temporal polyethism organises the division

of labour. A final aim is to investigate the effects of alarm pheromones on termites, at both an individual and colony level.

2 Chemical characterization of 3 major exocrine glands in the gaster of *Lasius flavus*

2.1 Abstract

The gasters of formicine ants contain three major exocrine glands which exit from the acidopore situated at the posterior of the ant. Chemicals secreted from the acidopore are used by workers for a number of tasks, involving deterring attackers, and laying pheromone trails. The three glands are the poison gland, Dufour gland and hindgut. The study presented in this chapter investigates the chemical constituents of these three glands in the poorly studied Yellow Meadow ant, *Lasius flavus*, using liquid chemical extraction and gas chromatography-mass spectrometry. In total, 27 chromatographic peaks were detected across the three gland types; 20 originating from the Dufour gland and 7 from the poison gland. No peaks were detected as originating in the hindgut. The Dufour gland was found to contain a range of alkanes and alkenes and a series of lactones. The chemicals detected in the poison gland constituted a series of carboxylic acids. This work confirms previous studies on the glandular contents of other *Lasius* species and more widely, other formicine ants. The presence of novel, high-concentration lactones detected in the Dufour gland suggests a potential antimicrobial function which has not yet been investigated. The same chemicals were often detected in all three gland types, this cross contamination (despite the stringent measures taken to prevent it) highlights the need to study the chemistry of neighbouring glands comparatively, rather than in isolation.

2.2 Introduction

As discussed in Chapter 1, the chemical ecology of *Lasius flavus* has not been well studied. As a formicine ant, *L. flavus* does not possess the stinging apparatus associated with most aculeates, and instead secretes compounds from an acidopore which is an opening at the posterior point of the gaster. The acidopore is utilised in a number of different tasks, including laying trail pheromone and repelling threats from other organisms using poisonous chemicals. There are 3 major exocrine glands which exit from the acidopore (see diagram in Chapter 1): the poison gland; the Dufour gland; and the hindgut. Only one study has investigated the compounds present in the gaster of *L. flavus*; the compounds detected included large amounts of undecane, 4-hydroxyoctadec-9-enolide (now known as micromolide), the corresponding acid (4-hydroxyoctadec-9-enoic acid) and a number of other alkanes and alkenes at low concentrations (Bergström and Löfqvist, 1970). The glandular sources of these compounds were not identified.

In formicine ants the poison gland is attached to a thin-walled poison reservoir, and although the number of publications which have examined the contents of this reservoir is small, in all cases the major component of the secretion was found to be formic acid (Bradshaw *et al.*, 1979; Chen *et al.*, 2013; Abraham Hefetz and Blum, 1978; A. Hefetz and Blum, 1978; Regnier and Wilson, 1968). In addition to this, smaller quantities of acetic acid have also been detected in the poison gland reservoir (Tragust *et al.*, 2013). Lopez *et al.* (1993) studied five species of formicine ant (*Anoplolepis custodiens, Camponotus vagus, Formica polyctena, Lasius niger and Polyrhachis gagates*) and found hexadecanol along with its formate and acetate in the poison

gland itself, but not in the reservoir. They hypothesized that these compounds protect the glandular tissue from the corrosive acids that the gland produces. Given that the closely related *L. niger* was examined in this study, we may expect to find hexadecanol in the poison gland of *L. flavus*. Formic acid is used as a very effective defence against other arthropods, and *L. neglectus* also use it to sterilize their pupae of fungal spores (Tragust *et al.*, 2013). Tranter *et al.* (2013) and Graystock and Hughes (2011) also found that *Polyhrachis dives* use their venom glands to treat fungal infections, and when their acidopores were blocked their ability to treat fungal infections was hampered. A novel use of formic acid was discovered by LeBrun *et al.* (2014); the tawny crazy ant *Nylanderia fulva* grooms itself with formic acid after being stung by the fire ant, *Solenopsis invicta.* This allows the crazy ant to detoxify the fire ant venom, and thereby outcompete the fire ant in its invasive range in the Southern States of the USA. How the detoxification occurs has yet to be determined, but LeBrun *et al.* (2014) hypothesized that formic acid denatures enzymes present in fire ant venom which are crucial to its insecticidal effects.

The Dufour gland is the most heavily studied of the three glands in formicine ants and although there is some variation in the compounds present in different species, undecane is always present in relatively large (µg) amounts alongside a series of other straight chain alkanes and alkenes (Hölldobler and Wilson, 1990). Lloyd *et al.* (1989) investigated the contents of the Dufour gland in 14 species of formicine ant in the genus *Myrmecocystus.* Alkanes, alcohols and ketones were found in all species, and tridecyl esters were found in two species of the subgenus *Eremnocystus.* The Dufour glands of *Nylanderia fulva* contain the expected alkanes and alkenes as well as large

quantities of 2-tridecanone (Chen *et al.*, 2013). A study investigating the Dufour gland contents of *Camponotus atriceps* and *C. floridanus* detected 37 components in the secretion. As expected, undecane was the most abundant compound, and other compounds were straight chain and branched alkanes and alkenes. In addition, 2,3-dihydrofarnesal and 9-octadecanol were also found in *C. floridanus* (Haak *et al.*, 1996). Bagnères *et al.* (1991) detected the usual hydrocarbons present in the Dufour glands of *Formica selysi, F. rufibarbis and C. lateralis*. In addition *F. rufibarbis* contained a large quantity of decyl acetate and the most abundant component of the *C. lateralis* secretion was tetradecyl acetate. In *L. niger* the Dufour gland was found to contain 50 components, including the commonly found alkanes and alkenes alongside more novel alcohols, acetates and propionates. Undecane and dodecyl acetate were the most abundant components (Attygalle *et al.*, 1987).

When investigating all the studies of Dufour gland secretions in species of formicine ants a general pattern emerges whereby undecane is always produced in large quantities and (in most cases) a ketone or ester is also found in a high abundance. These compounds are always accompanied by a large number of other hydrocarbons (usually straight chain alkanes and alkenes) found at far lower abundances. Occasionally a number of other hydrogenated compounds such as alcohols are also found. The purpose of these Dufour gland secretions is debatable. Some sources state that undecane and long chain esters and acetates may be used as wetting agents to aid the spread of formic acid; others suggest that high abundance ketones are themselves toxic (Chen *et al.*, 2013). By further investigating the compounds present in the Dufour

glands of other species of formicine ants, the use of Dufour gland compounds may become clearer.

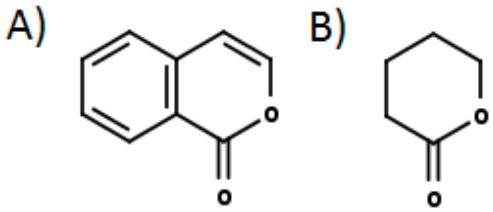

Fig. 1 – The two basic structures of all Formicine-ant pheromones discovered to date. **A)** Isocoumarin **B)** δ-lactone.

The hindgut in formicine ants forms the final part of the digestive tract and is covered in a number of small papillae which may act as a site where non-diet related compounds are synthesized (Hölldobler and Wilson, 1990). So far the trail pheromone of all studied formicine ants has been located in the hindgut and *L. flavus* follows this trend (Jones, 2014). Previous work has identified the trail pheromones of 16 formicine ants (Morgan, 2009). However, these trail pheromones have been particularly difficult to identify due to the relatively minute amounts of trail pheromone stored in the formicine hindgut. Many myrmicine and ponerine ant glands contain 1-100ng of trail pheromone, whereas in the formicine ants this value has never been more than 200pg (Morgan, 2009). All identified formicine trail pheromones are based on one of two potential chemical structures; either an isocoumarin structure (Figure 1A) or a δ-lactone structure (Figure 1B) (Morgan, 2009). Two species in the genus *Lasius* have had their trail pheromones identified. In *Lasius niger* the trail pheromone was identified to be *(R)*-3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (Bestmann *et al.*, 1992), and in *Lasius fuliginosus* it

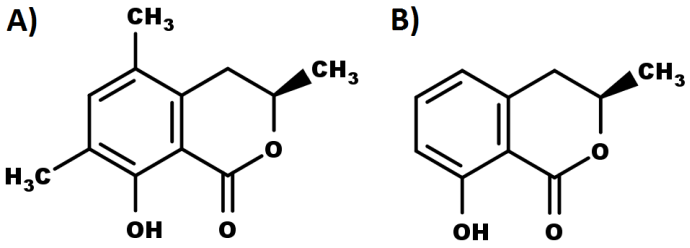

Fig. 2 – The structures of trail pheromones discovered in *Lasius* ants. A) *(R)*-3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin; the trail pheromone of *L. niger.* B) *(R)*-Mellein; the trail pheromone of *L. fuliginosus.*

was *(R)*-mellein (Kern *et al.*, 1997). The structures for these compounds are shown in Figure 2A and 2B respectively. In both studies trail following activity was confirmed by testing with pure standards.

The aim of this study is to identify the chemical contents of the poison gland, Dufour gland and hindgut of *L. flavus*. This information will provide further data regarding the specific profiles of compounds peculiar to each of the three studied glands of formicine ants, and potentially further elucidate the function of the Dufour gland in formicine ants. I also aim to confirm the glandular sources of the compounds identified by Bergström and Löfqvist (1970).

2.3 Materials and Methods

2.3.1 Chemicals

All solvents were HPLC grade and were purchased from Rathburn Chemicals Ltd, UK. All other chemicals were purchased from Sigma Aldrich, UK.

2.3.2 Ants

Three *L. flavus* colonies were collected on the University of Sussex campus and nearby Stanmer Park in in June 2012. *L. flavus* form mounds in open grassland so colonies were located by simply looking for mounds. Colonies were dug out of the ground and ants were separated from the soil by spreading out the collected material into a plastic tray and placing a number of partially water-filled test tubes (sealed with cotton wool) in the soil. The ants moved into the test tubes as the soil dried out and were subsequently transferred into a clean container. The containers were made of plastic

(40x30x20cm) and filled with plaster of paris to a depth of 2-3cm. The walls were coated in Fluon to prevent escape and a small amount of the soil in which the ants were collected was added as a foraging substrate. The containers also contained petri dishes filled with soil which the ants used as nests. All collected colonies were queenless. Colonies were kept in a temperature controlled room at 24°C on a natural day/night cycle and were fed on *Tenebrio molitor* larvae, *Drosophila melanogaster* and a 1:2 honey:water mixture. Water was provided *ad libitum.*

2.3.3 Gland dissection & extraction

Glands were dissected from a randomly selected sample of ten foraging ants from one of three captive colonies. Dissections were performed in air to prevent the dissolution of glandular contents which may occur in a liquid dissection medium; this also aimed to prevent the compounds from one type of gland contaminating another. To dissect out the glands, the rearmost tergite of the ant gaster was gently pulled with forceps until the poison gland, Dufour gland and hindgut were extruded. The forceps were cleaned with hexane between each gland dissection to minimize cross-contamination. Each gland was then immediately placed into a vial containing 30µl hexane and 1ng of 4,4'-dichlorobenzophenone (DCBP) as an internal standard. Separate vials were used for each gland type. The vials were kept on dry ice to prevent evaporation of the solvent. This was repeated ten times for each gland type, so each vial contained a composite sample of 10 glands. The vials were then placed into a sonic bath for 5 minutes to disrupt the glandular tissues. In order to concentrate any glandular compounds in the sample, the solvent was blown down to approximately 2µl under oxygen free nitrogen (OFN). The whole procedure was repeated three times in total

with each repeat using a sample of ten ants from one of the three captive colonies, producing three replicate composite samples for each gland type. Work up blanks were also produced for each of the repetitions using the same method without including any ant material. An aliquot (1µl) of each sample extract was analysed via GCMS, resulting in approximately 5 gland equivalents and 0.5ng of DCBP injected directly onto the GCMS column.

2.3.4 Chemical analysis

Sample extracts were injected in splitless mode on a Thermo Trace GC Ultra linked to a Thermo ITQ 1100 mass spectrometer. Helium was used as a carrier gas at a flow rate of 1.3ml/min. The column used was an Agilent DB-5ms Ultra Inert, 30m x 0.25mm internal diameter with a 0.25 µm film thickness. The GC oven was set to the following temperature programme: hold at 60°C for 4 minutes, ramp to 300°C at 10°C/min, hold at 300°C for 10 minutes. The mass spectrometer was used in electron ionization (EI) mode (70eV) with a scanning range between m/z 40 and 650. Chromatograms were analyzed using Thermo Xcalibur software (Thermo Xcalibur 2.3 build 26) and compounds were tentatively identified by comparison with the NIST/Wiley database (NIST MS Search 2.0 g). Where possible, identifications were confirmed by comparing the mass spectra and retention times of peaks with synthetic standards. Compounds were only identified as unique to a particular gland if they were not present in similar abundances in the other gland types. Any compounds present in the work-up blank were excluded from analyses as they were most likely to be contamination from external sources rather than compounds of interest.

Peaks were semi-quantified by calculating their area on a total ion chromatogram (TIC) relative to the TIC peak area of the internal internal standard (DCBP), therefore data in this study are provided in ng DCBP equivalents.

Kovat's retention indices (KI) were calculated for some compounds (e.g. alkenes) to assist with identification. To calculate the KI of a peak, a series of straight chain hydrocarbons containing all alkanes from heptane to triacontane was injected onto the GCMS instrument using the same temperature program as above. The retention times were noted for each hydrocarbon and the following formula was then used to calculate a KI for each peak:

$$I = 100 \times [n + \frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}}$$

Where:

I = Kovat's retention index

n = the number of carbon atoms in the alkane which elutes before target peak

N = the number of carbon atoms in the alkane which elutes after target peak

t_r = retention time of: *(unknown)* – target peak; *(n)* – earlier eluting hydrocarbon; *(N)* – later eluting hydrocarbon

The KI values for unknown peaks were then used to find potential identities by interrogating databases for matching KI values in studies which used a GCMS column with a similar coating to the DB-5ms Ultra Inert coating used here. These databases included NIST/Wiley and Pherobase (http://www.pherobase.com).

2.4 Results

Typical TICs for each gland extraction and the workup blank are shown in Figure 2. In total 27 peaks were found over the three gland extractions which were not attributable to bleed peaks or work-up blank contamination. Many peaks were found in more than one chromatogram, indicating that cross-contamination did likely occur during the gland dissection and extraction process despite the steps taken to prevent it. There were no peaks detected which were completely unique to the hindgut. Peaks 4, 5, 8, 17, 22 and 24 were unique to the poison gland and peak 21 was largest in the poison gland extraction. All other labelled peaks were either unique to the Dufour gland or were largest in the Dufour gland extraction.

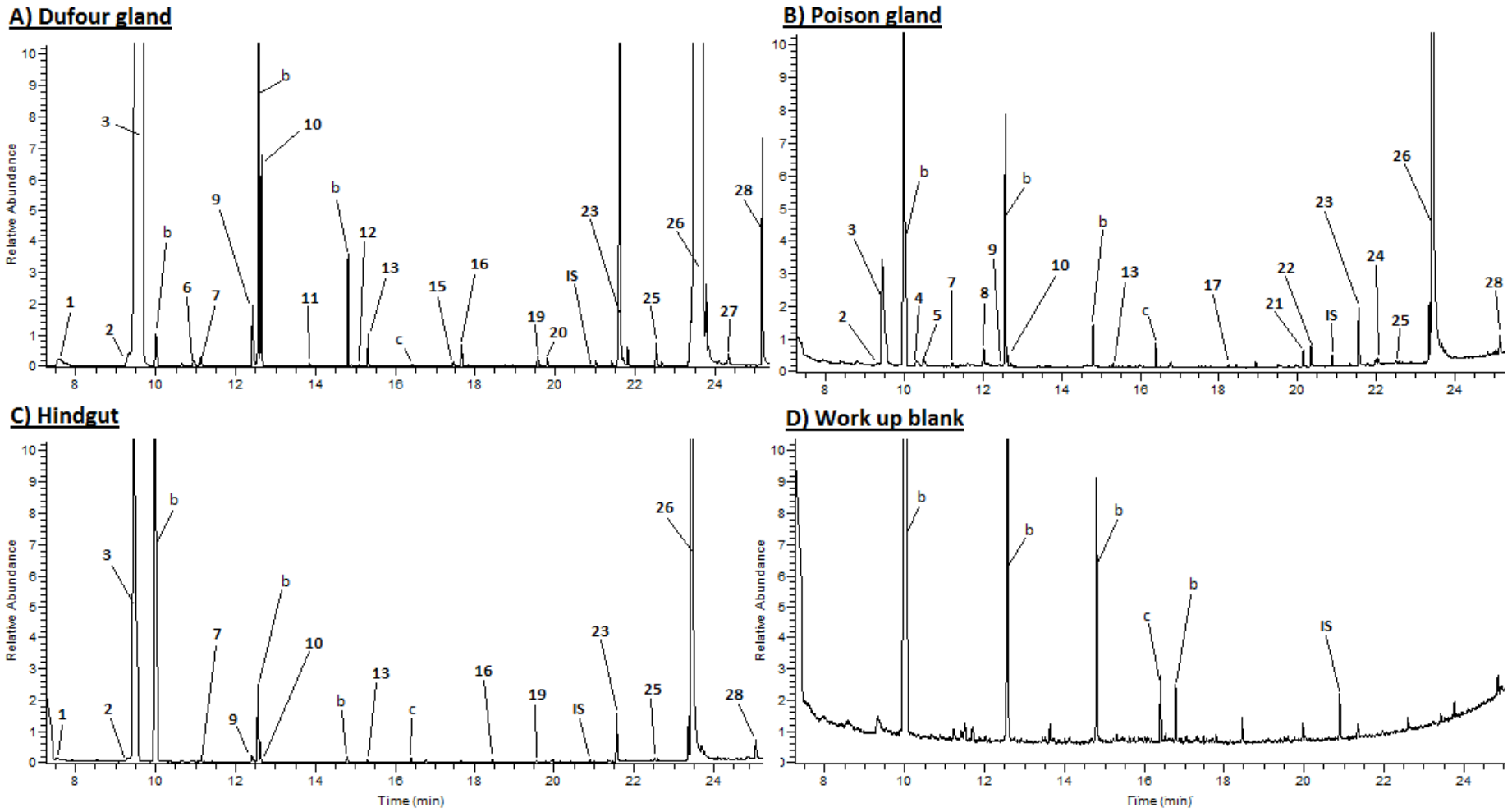

Figure 3 – Representative total ion chromatograms shown at 10x magnification for **A)** an extraction of Dufour glands, **B)** an extraction of poison glands, **C)** an extraction of hindguts from *Lasius flavus* workers and **D)** a work up blank containing no ant material. Approximately 5 gland equivalents were injected onto the GCMS column. Peaks are numbered and some are visible in more than one chromatogram (e.g. 26). Some peaks are contaminants from the workup (c), or are polysiloxane bleed peaks (b) from the GCMS column or inlet septa. The internal standard is labelled **(IS).**

2.4.1 Compounds identified in the Dufour gland extractions

A typical total ion chromatogram (TIC) of the Dufour gland extractions is shown in Figure 3A. The largest peaks in the Dufour gland extraction were peaks 3 and 26 found at retention times 9.59 and 23.56 respectively.

Peak 26 (RT 23.56 minutes) accounts for 56% of all peak areas present in the Dufour gland extraction and its average concentration was 3.7µg DCBP equivalent per Dufour gland whereas concentrations of 77ng and 30ng DCBP equivalent were detected per hindgut and poison gland respectively. This strongly indicates that peak 26 originates from, or is stored in, the Dufour gland. The mass spectrum did not match any compounds in the databases used, but it did match the spectrum for the γ-lactone micromolide published by Bergström and Löfqvist (1970) and more recently by Herzner *et al.* (2013). The spectrum for peak 26 and the published micromolide spectrum are compared in Figure 4 and show a similar array of ion fragments. The molecular ion is present at m/z 280, and the first loss in the spectrum is m/z 18 which is a loss of H_20 from the lactone ring. The next peak on the spectrum is m/z 220, which corresponds to a loss of m/z 60. This is a loss of $C_2H_4O_2$ as a result of the cleavage of two bonds in the lactone ring; these cleavages are shown in the insert in Figure 4. The sequence of losses of m/z 14 and m/z 15 (leading to the visible ions 136, 122, 121, 108 etc.) are caused by fragmentation along the hydrocarbon chain and represent the loss of CH_2 and CH_3.

The nearby peaks in the TIC; 23, 25, 27 and 28 all share the same fragmentation pattern but their respective molecular ions are m/z 252, 266, 294 and 308. This

indicates that they are homologues of micromolide, each containing a different number of carbon atoms. Peak 23 contains 16 carbons, peak 25 contains 17, peak 266 (micromolide) contains 18, peak 27 contains 19 carbons and peak 28 contains 20. Mass spectra for each of these peaks can be seen in Appendix I at the end of this thesis.

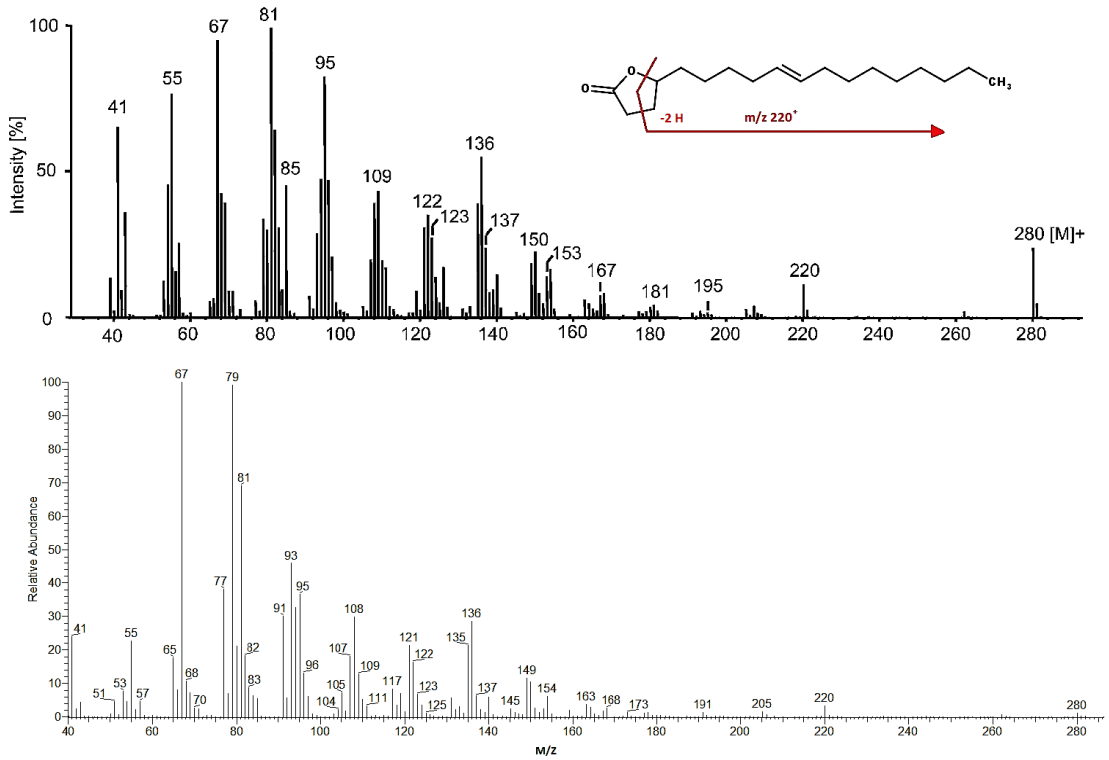

Fig. 4 – A comparison of mass spectra indicating that micromolide was present at peak 26 in the Dufour gland extraction. *Top*: Mass spectrum of micromolide from Herzner *et al.* (2013); *Bottom*: Mass spectrum of peak 26 from the Dufour gland extraction.

The mass spectrum of another major compound detected in the extract of Dufour glands, peak 3 (RT 9.59 mins), very closely matches that of undecane. A comparison between peak 3 in the Dufour extraction and a synthetic undecane standard is shown in Figure 5. Both the retention times and mass spectra of the standard and peak 3 closely matched. The fragmentation pattern of undecane is unclear when looking at the higher molecular weights, but from m/z 85 and below the pattern is characteristic of a straight chain hydrocarbon. The sequential losses of m/z 14 to produce ions 85, 71, 57 and 43 are clearly visible. This pattern is caused by the loss of CH_2 from the hydrocarbon chain of the molecule. The mean concentration of undecane per Dufour gland was 2.6µg DCBP equivalent, making up 39% of the mass of all compounds in the Dufour gland extraction. A series of seven other hydrocarbons were also detected in much lower abundances (<50ng DCBP equivalent per gland), peaks 1, 7, 10, 13, 16 and 20 in Figure 3. These peaks had fragmentation patterns identical to that of peak 4. The identities of these compounds were confirmed by comparing retention times and mass spectra which matched those of a standard hydrocarbon series. Extracted ion chromatograms for m/z 57 (a characteristic ion of alkanes) for a Dufour gland extraction and the alkane series standard are shown in Figure 6. In all, eight

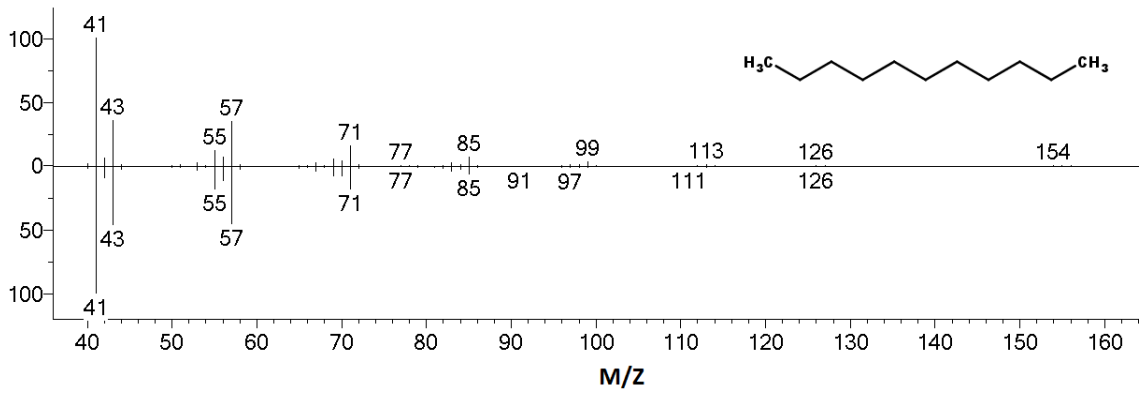

Fig. 5 – A comparison of mass spectra indicating that undecane was present at peak 3 in the Dufour gland extraction. *Top:* a synthetic standard of undecane; *Bottom:* peak 3 from the Dufour gland extraction.

hydrocarbons were identified in the Dufour gland extraction, n-C10, n-C11, n-C13, n-C14, n-C15, n-C16, n-C17 and n-C19.

Seven alkenes were also detected in the Dufour gland extraction (peaks 2, 6, 9, 11, 12, 15 and 19 in Figure 3). The average abundance of these compounds were all below 20ng DCBP equivalent per gland. The mass spectra of all these compounds matched the characteristic pattern found in all straight chain alkenes, with fragment ions of m/z 41, 55, 69, 83 and 97. The consecutive losses of 14 represent CH_2 being lost from the carbon chain of the molecule, as is the case with alkanes. The molecular weights of alkene fragments are 2 m/z lower than alkane fragments due to the double bond which exists between two carbon atoms; this results in the molecule containing 2 fewer hydrogen atoms than the equivalent alkane. Kovats Retention Indices (KI) were calculated for these peaks and compared to published data to assist with identification. The closest matching published KI for an alkene on a GCMS column with a coating similar to that used in this chapter (DB-5ms UI) was found for each peak. The calculated KIs for the alkenes in the Dufour gland along with the closest matching published KIs are shown in Table 1. KI data were not available for all isomers of each alkene, therefore the exact position of the double bond cannot be deduced.

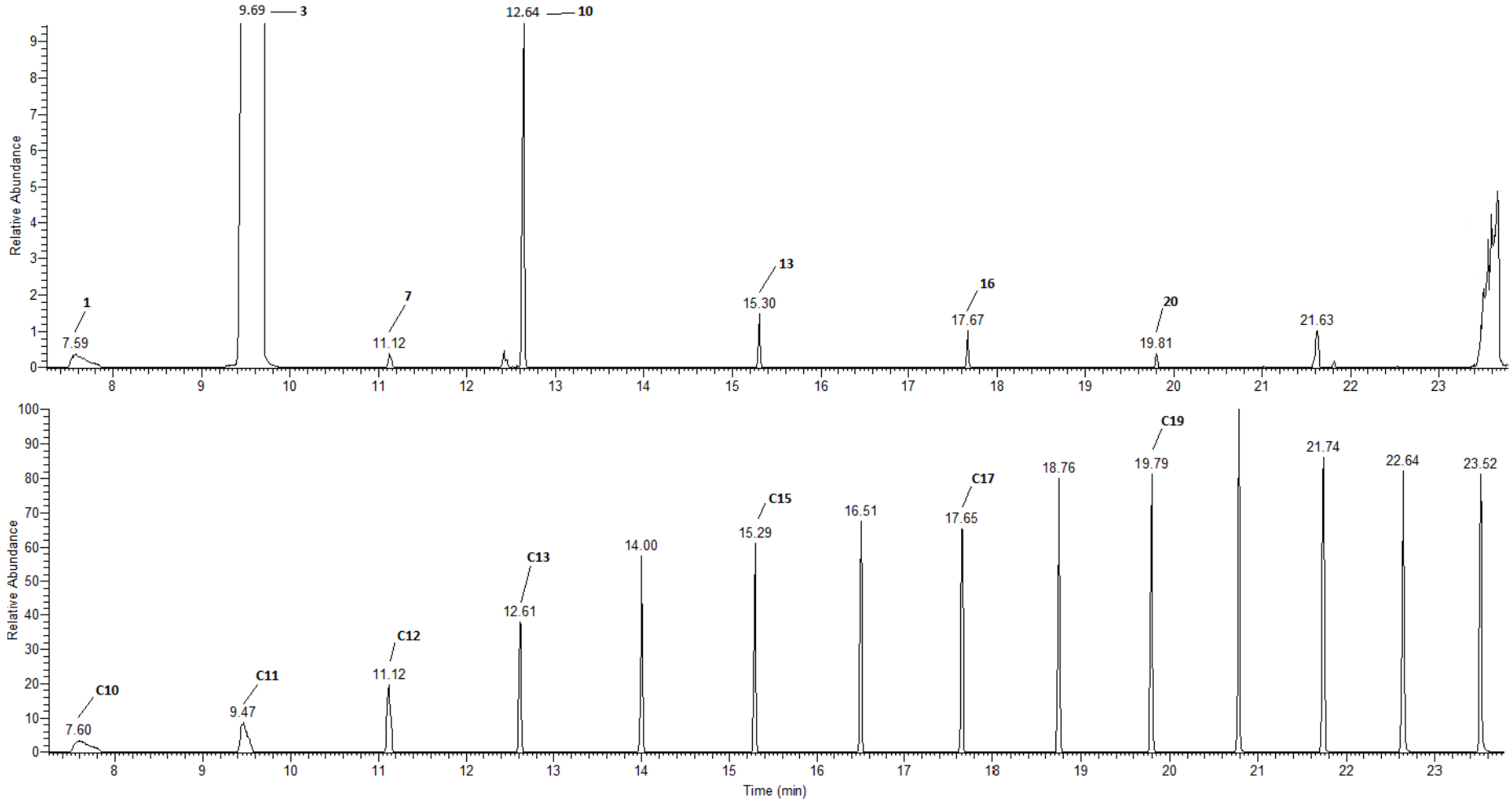

Fig. 6 – A set of alkane standards confirm the presence of alkanes in the Dufour gland extraction. *Top* – An extracted chromatogram for m/z 57 (characteristic of alkanes) from the Dufour gland extraction (10x zoom), peak labels correspond with those in figure 2. A trace peak was also present at retention time 16.51 minutes but is not visible on this chromatogram; *Bottom* – A series of straight chain hydrocarbon synthetic standards, those with matching peaks in the Dufour gland extraction are labelled with the number of carbons.

#	RT	Calculated KI	Closest matching KI	ID	Reference
2	9.32	1093	1092	1-Undecene	Kohl, E. *et al.* (2001)
6	10.95	1190	1190	1-Dodecene	Hölldobler, B. *et al.* (2004)
9	12.41	1287	1287	6-Tridecene	Takeoka, G. *et al.* (1996)
11	13.84	1389	1389	1-Tetradecene	Binder, R.G. *et al.* (1990)
12	15.06	1483	1481	1-Pentadecene	Heinze, J. *et al.* (1998)
15	17.44	1682	1679	8-Heptadecene	Heinze, J. *et al.* (1998)
19	19.57	1880	1879	7-Nonadecene	Stránský, K. *et al.* (2001)

Table 1 – The alkenes found in the Dufour gland extraction along with calculated Kovat's Indices (KI) and the closest matching published KI; the close matches indicate that these alkenes were present in the extraction. As KI data are not available for all isomers of alkenes the exact position of the double bond cannot yet be deduced.

2.4.2 Compounds identified in the poison gland extractions

The chromatogram for the poison gland extraction is shown in Figure 3B and as was the case with the Dufour gland extraction, the largest (non-bleed) peaks are 3 and 26. The peaks had the exact same mass spectra as those found at the corresponding retention times in the Dufour gland extraction. They were far less abundant in the poison gland extraction and were found at 6.5 and 30.5ng DCBP equivalent; this is over 100x less than that found in the Dufour gland. This indicates that the presence of these peaks is probably due to cross contamination of undecane and micromolide from the Dufour gland.

Seven compounds were detected in the GCMS data for the poison gland extractions which were not found at a similar abundance in the other gland extractions and all of them were present in quantities lower than 1ng DCBP equivalent. Six of these compounds were carboxylic acids. The most abundant carboxylic acid found was hexadecanoic acid, which is peak 22 in Table 2. The retention time of hexadecanoic acid was 20.36 minutes, and the mean amount found per gland was 0.128ng DCBP equivalent, which accounts for almost 20% of detected poison gland secretion. The other carboxylic acids were all aliphatic apart from peak 5 which has a mass spectrum

matching that of the aromatic compound, benzoic acid. The identities of all carboxylic acids were confirmed by comparing their retention times and mass spectra with those of synthetic standards, mass spectra are shown in Appendix II at the end of this thesis.

The only compound detected in the poison gland extraction that was not a carboxylic acid was peak 21. This was also the most abundant compound identified in poison gland extractions with a mean abundance of 0.23ng DCBP equivalent per gland. This compound was difficult to identify as there is no clear molecular ion and the fragmentation pattern is rather ambiguous. The mass spectrum is shown in Figure 7.

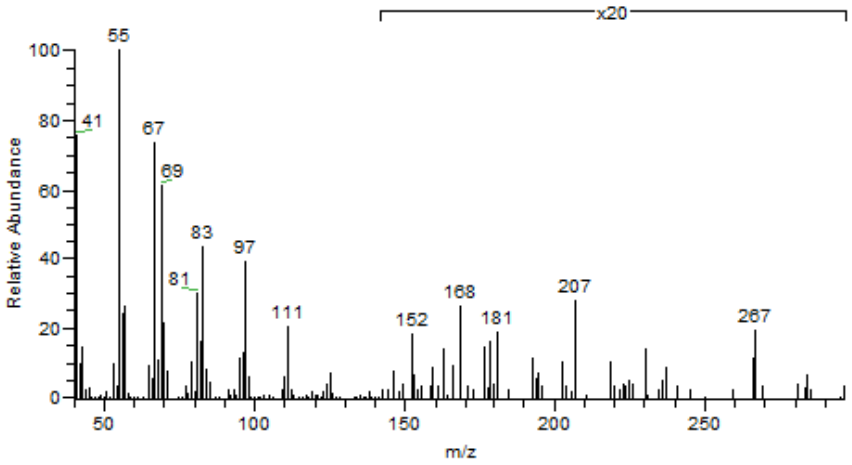

Fig. 7 – The ambiguous mass spectrum for peak 21. There is no clear molecular ion and the spectrum did not match any from the standard databases.

The pattern of ions 41, 55, 67, 69, 81, 83, 97 and 111 could belong to a cyclic alkane, an alkene (although this is unlikely based on the calculated KI for this compound, 1940, which does not match any published KI for alkenes), an alcohol, an aldehyde or an ester. As such, it is difficult to assign even a tentative identification to this peak.

A summary of all compounds found and identified in the gland extractions of *L. flavus* workers along with their abundance relative to the DCBP internal standard is shown in Table 2. The relative abundance is shown as amount per gland in nanogram DCBP

equivalents. Many compounds were present in two or more types of gland extraction and the most likely explanation for this is cross contamination during dissection, despite the steps taken to avoid this. Because of this contamination, the gland in which each compound most abundant was identified as the probable gland of origin. Overall 20 compounds were detected that originated in the Dufour gland, 7 compounds were detected that originated in the poison gland and no compounds were detected which appeared to originate in the hindgut.

#	RT (Minutes)	ID	Poison Gland Abundance	Dufour Gland Abundance	Hindgut Abundance	Likely gland of origin	Kovats Index
1	7.59	Decane*		10.6 ± 2.72	0.065 ± 0.113	Dufour	1000
2	9.32	Undecene	0.047 ± 0.081	14.3 ± 5.12	0.283 ± 0.489	Dufour	1092
3	9.59	Undecane*	6.51 ± 7.91	2570 ± 1066	31.1 ± 53.4	Dufour	1100
6	10.95	Dodecene		1.96 ± 0.529		Dufour	1190
7	11.12	Dodecane*	0.007 ± 0.012	3.56 ± 1.24	0.027 ± 0.047	Dufour	1200
9	12.41	Tridecene	0.036 ± 0.063	19.1 ± 4.54	0.174 ± 0.301	Dufour	1287
10	12.62	Tridecane*	0.205 ± 0.094	46.3 ± 11.1	0.488 ± 0.799	Dufour	1300
11	13.84	Tetradecene		0.615 ± 0.112	0.009 ± 0.016	Dufour	1388
12	15.06	Pentadecene		0.184 ± 0.045		Dufour	1482
13	15.3	Pentadecane*	0.029 ± 0.027	5.08 ± 1.11	0.05 ± 0.047	Dufour	1500
14	16.51	Hexadecane*	0.011 ± 0.009	0.063 ± 0.014	0.015 ± 0.014	Dufour	1600
15	17.44	Heptadecene		0.668 ± 0.145	0.006 ± 0.01	Dufour	1681
16	17.66	Heptadecane*	0.009 ± 0.001	3.1 ± 0.726	0.03 ± 0.034	Dufour	1700
19	19.57	Nonadecene		1.66 ± 0.503	0.023 ± 0.04	Dufour	1879
20	19.81	Nonadecane*		1.33 ± 0.354	0.011 ± 0.019	Dufour	1900
23	21.59	C16 Lactone	0.641 ± 0.667	131 ± 48.4	1.12 ± 1.71	Dufour	2084
25	22.52	C17 Lactone	0.021 ± 0.037	4.45 ± 1.07	0.059 ± 0.103	Dufour	2186
26	23.56	Micromolide	30.5 ± 25.8	3678 ± 1259	77.3 ± 121	Dufour	2305
27	24.31	C19 Lactone	0.007 ± 0.012	2.04 ± 0.483	0.02 ± 0.035	Dufour	2395
28	25.16	C20 Lactone	0.382 ± 0.312	41.7 ± 8.59	0.414 ± 0.605	Dufour	2500
4	10.31	Benzoic acid*	0.054 ± 0.058			Poison	1151
5	10.5	Octanoic acid*	0.069 ± 0.047			Poison	1163
8	12.02	Nonanoic acid*	0.11 ± 0.092			Poison	1254
17	18.26	Tetradecanoic acid*	0.015 ± 0.013			Poison	1755
21	20.18	Unknown	0.232 ± 0.187		0.013 ± 0.006	Poison	1939
22	20.36	Hexadecanoic acid*	0.128 ± 0.097			Poison	1957
24	22.05	Octadecanoic acid*	0.038 ± 0.036			Poison	2134

Table 2 – Compounds identified in *Lasius flavus* gland extractions. Identifications are tentative unless marked with a *; these peaks have had identifications confirmed by comparing mass spectra and retention times with synthetic standards**.** Abundances are expressed as the mean ± standard deviation per gland in ng DCBP equivalents, n = 3 colonies. No compounds were detected which originated in the hindgut. The number in the # column corresponds with the peak labels in Figure 3.

2.5 Discussion

In total 24 peaks were analysed from the three gland extractions. 23 of these peaks were tentatively identified using database comparisons. 14 of those tentative identifications were subsequently confirmed by comparing retention times and mass spectra with synthetic standards. Many compounds were present in more than one type of gland extraction, however by comparing the abundance of compounds in different extractions it becomes clear what the glandular source of each compound is. This overlap of chemical profiles between different gland extractions can be explained by cross contamination during the dissection procedure, despite the careful measures taken to avoid this. Many previous studies of gland contents have only analysed a single gland type (Attygalle et al., 1987; Bagnères et al., 1991; Hölldobler et al., 2013; Lloyd et al., 1989; Witte et al., 2007a), but the results of this study highlight the need to perform a comparative analysis to ensure that the glandular sources of identified compounds are correctly identified.

The most abundant compounds identified in the Dufour gland extraction were the γ-lactone micromolide and undecane. This matches the pattern of compounds identified in the Dufour glands of other formicine ants, where undecane is always found in large amounts and is thought to act as a spreading agent for formic acid (Bergström and Löfqvist, 1970; Francke *et al.*, 2014; Löfqvist, 1977). It is often accompanied by a high abundance ketone or ester. Undecane is a compound which has been identified in many different species of insect (Howard and Blomquist, 1982), but micromolide is much rarer and has only been identified in three other organisms. The compound was first identified in whole extracted gasters from *Lasius flavus* (Bergström and Löfqvist,

1970) but its use was never investigated. It was also identified in the currant stem girdler, *Janus integer,* as a female specific pheromone (Cossé *et al.*, 2001). Ma *et al.* (2005) found the compound in the bark of the plant *Micromelum hirsutum*, giving it the name micromolide and suggesting that it is formed as an octadec-9-enoic acid (oleic acid) derivative. The authors also found that micromolide was very effective at inhibiting the growth of *Mycobacterium tuberculosis* (TB) bacteria. The larvae of the emerald cockroach wasp, *Ampulex compressa*, also produce micromolide which is used (alongside other compounds) as an antibacterial compound to disinfect the cockroach bodies which the larvae live in and feed on (Herzner *et al.*, 2013). The compounds identified in the Dufour gland of formicine ants have been thus far considered to be used as wetting agents for formic acid application or defensive compounds against arthropod attackers. However, the presence of micromolide in the Dufour glands of *Lasius flavus* indicate a potential third use; that of disinfectant compounds. It may be the case that Dufour gland compounds are used to disinfect the ant nest, brood and food. It has already been established that formicine ants use their acid to prevent the germination of fungal spores on their brood and in their nest (Tragust *et al.*, 2013) and Dufour gland compounds may be used to further increase the effectiveness of this behaviour. Alternatively these compounds may be used strictly as antibacterial compounds to complement the antifungal effect of formic acid. Formicine ants lack the metapleural gland possessed by other superfamilies of ant and this gland is usually the source of antibiotic compounds (Hölldobler and Wilson, 1990). It has been suggested that formicine ants use their venom in place of the metapleural gland (Graystock and Hughes, 2011), but perhaps given the results here, the Dufour gland may also play a part in replacing the function of the metapleural gland. Many of the homologues of

micromolide detected here in *L. flavus* Dufour glands have never been detected before. The C16 homologue, 4-hydroxyhexadec-9-enolide, was identified in *Desmocerus californicus* beetles and is used as a female-produced sex pheromone and was also found in *L. flavus* gasters (Bergström and Löfqvist, 1970). The other homologues (C17, C19 and C20) have never been recorded in the literature, although their extremely low abundance relative to micromolide indicates that they may be byproducts created during the synthesis of micromolide.

Given the results of previous research on the compounds present in the Dufour gland of formicine ants, it is unsurprising that a range of alkanes and alkenes were also detected in the Dufour gland in this study. The high abundance of undecane is also unsurprising, and as has been suggested this compound is likely to be a wetting agent to assist in the application of formic acid as a defensive substance. Some work has suggested that undecane may be a component of the alarm pheromone of formicine ants. However extractions of *L. flavus* Dufour glands do not attract foraging workers when presented on a T-maze (Jones, 2014). This makes the proposed alarm function of undecane unclear in *L. flavus*, and more work must be done to confirm whether the alarm effect is present in this and other species.

All compounds found by Bergström and Löfqvist (1970) in *L. flavus* gasters originate from the Dufour gland. The only compound not detected was the acid 4-hydroxyoctadec-9-enoic acid. Perhaps this acid was formed as an artefact from the extraction or storage techniques used, or the non-polar column used here may not have been suitable for analysis of long chain carboxylic acids with an additional hydroxyl group due to excessive compound retention.

The poison gland contained six carboxylic acids and one unidentified peak. Formic and acetic acid were not detected here, but this is probably due to the analytical methods used rather than its absence in the gland, as the odour is clearly present when a poison gland is crushed. Formic acid is more volatile than many commonly used solvents, so it can be difficult to detect using liquid extraction techniques on non-polar GCMS columns such as the type used here. Previous studies have used wax GCMS columns and solid phase micro extraction (SPME) (Tragust *et al.*, 2013) or solid sample, solvent free injection (Bradshaw *et al.*, 1979) to analyse formic acid in formicine ant poison glands.

Benzoic acid has never before been found in a Formicine ant, although it has been identified in the male accessory glands of *Solenopsis invicta* (Mikheyev, 2003) and the pygidial glands of *Messor pergandei* (Hölldobler *et al.*, 2013) but its function remains unclear. A mixture of carboxylic acids, including octanoic acid and nonanoic acid (along with others) was initially identified as the trail pheromone of *Lasius fuliginosus* (Huwyler *et al.*, 1975). However this function was later disproven and the actual trail pheromone compound was identified as mellein (Kern *et al.*, 1997). Tetradecanoic acid was supposedly detected in the Dufour glands of the formicine ant *Paratrechina longicornis* (Witte et al., 2007a), however a comparative analysis of other glands was not performed so this is not certain. Hexadecanoic acid was found in the poison gland of *Camponotus pennsylvanicus* workers (Hillery and Fell, 2000) but its function was not determined. Hexadecanoic acid and octadecanoic acid were detected in the postpharyngeal glands of *Formica japonica* where they were proposed to be contact pheromones on the ant cuticle used as nestmate recognition recognition cues along

with cuticular hydrocarbons (Akino *et al.*, 2004). The study of the contents of formicine poison glands has been relatively neglected, and only with further analysis of other species can the role of these carboxylic acids be determined.

It is disappointing that no compounds were detected as originating from, or unique to the hindgut, as this gland is where the trail pheromone of *L. flavus* appears to be produced and/or stored (Jones, 2014). This may be because any chemicals present are simply at concentrations too low to be detected by conventional methods, that they may be too volatile for the methodology used here, or that they may be too polar and did not elute from the non-polar column used in this study. Further work will be necessary to identify the trail pheromone of *L. flavus,* perhaps by utilizing larger and more concentrated extractions, or by performing solvent free extractions to prevent dilutions of analytes.

3 The right tools for the job: Temporal Biochemical Polymorphism in *Lasius niger*

3.1 Abstract

Social insects use division of labour to organise the work performed by the colony. Many species of ants use a system known as temporal polyethism to accomplish this, in which the tasks a worker performs varies depending on its age. *Lasius niger* is known to use this system; young workers perform nest-based tasks such as tending to brood and grooming the queen, while older workers perform riskier tasks outside the nest such as foraging for resources and repelling invaders. Ants also use chemicals when performing these tasks. Corrosive, poisonous and repellant chemicals are used to repel invaders and trail pheromones (found in the hindgut) are used to guide nestmates to sources of food. It has never been investigated whether the chemicals produced by an ant worker vary depending on the temporal caste they belong to. The work presented in this chapter investigates the chemicals present in the hindgut of *L. niger* workers of two different temporal subcastes; nurses and foragers. Liquid extractions of hindguts were analysed by GCMS and the data were investigated using multivariate models. Trail following bioassays of nurse and forager hindgut extracts were also performed to complement the chemical analyses. Foragers were found to follow trails of forager hindgut extractions further than they did nurse hindgut extractions, while nurses did not follow either type of extraction. The chemical analyses detected 14 compounds that were at higher concentrations in forager hindguts than nurse hindguts. These

included the trail pheromone, some compounds with the potential to be used to repel invaders and potentially a sesquiterpene related to their symbiotic aphid, *Aphis fabae.* Four compounds were detected at higher concentrations in nurse hindguts of which 3 could be assigned a tentative identification as amino acids. These amino acids may be a consequence of nurses sequestering more proteinaceous food to feed to larvae, or they may be used by nurses in the production of trophic eggs. These results indicate that the chemical tools possessed by a worker ant are tailored to the tasks performed by their subcaste.

3.2 Introduction

As described in Chapter 1, all ant species exhibit some form of division of labour. At the simplest level this division is limited to reproduction; a single queen ant reproduces while all additional labour (such as nest maintenance or foraging) is performed by other, often sterile, ants. In this scenario both reproductive and non-reproductive ants can be morphologically identical; this is the case in ants belonging to the genus *Dinoponera* where all colony members have the potential to reproduce, but only one 'alpha' female does (Monnin et al., 2002). A more complex division of labour is found in leafcutting ants in the genus *Atta.* Not only are the reproductive queens morphologically distinct from the non-reproductive workers, but the workers themselves are separated into at least four morphological castes. Each caste performs a different range of tasks, and the size and shape of the worker is suited to the tasks they perform. For example, the largest workers (majors) have larger heads proportional to their body size, with very powerful jaw muscles. These workers act as

soldiers, using their powerful bite to deter predators. The smallest workers (minims) are small enough to remove single hyphae from the fungus which the ants cultivate as a food source (Hölldobler and Wilson, 1990). Between these two levels of complexity is division of labour by temporal polyethism, where workers are monomorphic. This term describes a system where the tasks a worker primarily performs are determined by its age. Young workers tend to perform low risk tasks within the nest, such as tending to the queen and cleaning and feeding brood, whereas older workers primarily perform risky tasks outside the nest, such as scouting and foraging. This system produces the maximum amount of work effort over the lifetime of an individual worker and is found in almost all species of ant, even those with polymorphic workers (Hölldobler and Wilson, 1990).

Lasius niger is a well-studied species of ant which has monomorphic workers and utilizes temporal polyethism to organise work effort in its colonies (Hölldobler and Wilson, 1990). One behaviour that is affected by temporal polyethism is that of aggregation; young workers (brood-tenders, or nurses) aggregate close together to form dense, stable clusters whereas older workers do not (Depickère et al., 2004).

Ants are biochemical factories, each possessing many different exocrine glands which produce and store metabolites with a large potential range of functions (Billen, 2011). All species produce unique suites of pheromones and defensive substances which constitute a 'chemical toolkit'. The tools in the toolkit have a variety of functions. Some tools are defensive, such as alkaloids present in the venom of myrmicine ants (Morgan, 2008). Other tools are used to attract nestmate ants; trail pheromones are used in this way by many ant species to recruit nestmates to food sources (Morgan, 2009).

Chemical differences have been found between the different sexual castes in ants, with queens, males and workers of various species producing different glandular chemicals. Males of five different species of *Camponotus* produce 6-methyl salicylate, 2,4-dimethyl-2-hexenoic acid and methyl anthranilate, while no trace of these compounds can be detected in queens or workers (Brand et al., 1973). Recently queen pheromones have been discovered which inhibit worker reproduction and are only produced by ant, wasp and bee queens and not non-reproductive workers (Oystaeyen et al., 2014). Differences in glandular chemistry have also been detected between the distinct morphological castes found in some polymorphic species of ant. For example major workers of *Pheidole fallax* produce skatole in their poison glands, whereas the minor workers do not (Law et al., 1965).

Although behavioural differences caused by temporal polyethism in ant species with monomorphic workers have been demonstrated (Hölldobler and Wilson, 1990), the differences in the chemical toolkit of workers has never been investigated. The cuticular hydrocarbons of *Pogonomyrmex barbatus* were found to vary depending on the temporal caste they belonged to (Wagner et al., 1998), but this was later found to be due to environmental differences encountered by workers performing tasks inside or outside the nest (Wagner et al., 2001). Whether differences are present in the glandular toolkit of different temporal castes is yet to be established.

Lasius niger is a useful model organism to investigate whether a chemical difference exists between temporal castes. As explained above, measurable behavioural differences are present based on worker age which can be used to identify the temporal caste a worker belongs to. Furthermore, the trail pheromone of *L. niger* has

already been identified as 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (the structure is shown in Figure 1), a compound produced in the hindgut of the ant and one that is detectable using GCMS (Bestmann et al., 1992). Nurses do not create foraging trails; it therefore may be a waste of resources for a nurse to synthesize trail pheromone. If the hypothesis of a chemical toolkit customized to suit the tasks performed by a temporal caste is correct, I predict that the hindguts of nurses contain less trail pheromone than those of foragers, perhaps none at all. I also predict that any other chemicals present in solvent extractions of hindguts will be suited to the tasks performed by the temporal caste.

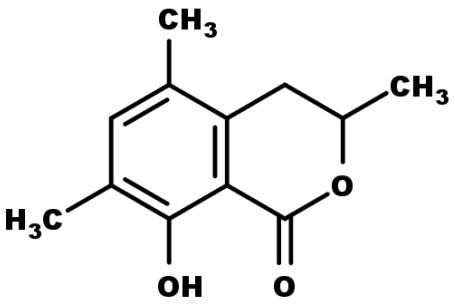

Fig. 1 – The chemical structure of the trail pheromone of *Lasius niger*, 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin.

3.3 Materials and Methods

3.3.1 Chemicals

All solvents were HPLC grade and all chemicals were purchased from Sigma-Aldrich, UK. *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and pyridine were used as a derivitising agent to perform silylation of functional groups in order to increase the volatility of polar extracted metabolites.

3.3.2 Collection and storage of ants

Six queenless *L. niger* colony fragments were collected from underneath paving slabs placed around the University of Sussex campus. Each fragment contained 1000-3000

ants along brood consisting of eggs, larvae and pupae. The ants were housed in the laboratory in plastic containers measuring 30cm x 30cm x 10cm and filled to a depth of 2-3cm with Plaster of Paris. The walls of the tubs were coated in Fluon to prevent escape. The containers also contained petri dishes filled with Plaster of Paris which had been carved to mimic natural nesting chambers. Colonies were kept under a natural light cycle and ambient temperatures ranging from 18-25°C. Ants were fed on a diet of *T. molitor* larvae, *D. melanogaster* and a 1:2 honey:water mixture. Water was provided *ad libitum*. Ants were kept in the lab for approximately 1 week before experiments began.

3.3.3 Identification of forager and nurse castes

Foraging ants were collected from outside the petri dish nest, and nurse ants were collected from those observed to be clumped on and around brood inside the nest. The identity of collected ants was then confirmed by placing them in a petri dish and leaving them for 60 minutes to assess aggregation behaviour. Ants forming aggregations (two or more individuals in a group separated by less than 1cm) were identified as nurses, and those which remained alone were identified as foragers (Depickère et al., 2004). If ants did not behave as expected (i.e. foragers forming aggregations or nurses not forming aggregations) they were excluded from the experiment.

3.3.4 Extraction and bioassay to test biological potency of hindguts from each caste

Extractions of hindguts from nurses and foragers were made and tested on a trail following bioassay to determine whether trails made from forager or nurse hindgut

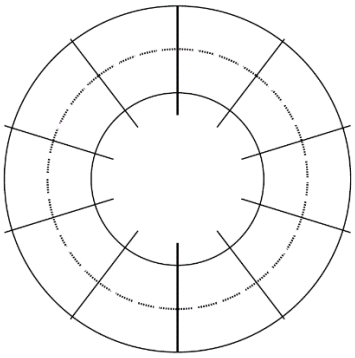

Fig 2. – The circular bioassay used to test potency of extractions at actual size. The radii intersect 1cm arcs on the centre (striped) concentric ring.

extracts possessed different potencies. 7 hindguts of each test caste were dissected in air and placed into 70µl of diethyl ether. The samples were kept on dry ice throughout the dissection to prevent the evaporation of solvent. Extractions were made from the hindguts by placing the vials in a sonicator bath (5 minutes) followed by a vortex mixer (1 minute). The liquid phase of the sample was then transferred into a new vial to ensure that the amount of time glands spent being extracted in solvent was the same between all samples. This extraction process was repeated twice for each of the 6 experimental colonies, producing 24 extractions in total (12 for each caste), with each extraction containing the hindguts from 7 workers. The circle assay described by Morgan (2009) was modified in order to create a rigorous assay which quantified the distance an ant follows a trail and to make sure the results were analysable using simple statistical tests. A diagram of the assay is shown in Figure 2; this was printed onto standard printer paper at a size where the distance of the arc of the dashed line between each of the marked radii was 1cm and the solid bounding lines were always exactly 0.5cm away from the dashed line. A test ant was selected at random from a random colony, then restrained in the middle of the centre circle using an open-ended plastic cylinder with fluon-coated internal walls. The ant was left to habituate for 30 seconds before a test trail of 10µl (1 gland equivalent at the extraction concentration detailed above) was laid evenly on the dashed circle using a Hamilton syringe, and the solvent allowed to evaporate for a further 20 seconds. The test ant was released and the number of 1cm arcs (of the dashed line) completely crossed was

recorded until the ant completely crossed one of the two solid bounding lines. If upon release the test ant did not move for one minute, or if they displayed alarm behaviour, they were excluded from the experiment. This was repeated for each sample until 10µl could no longer be drawn up from the vial, producing between 2 and 5 replicates for each individual extraction. The whole procedure was initially performed using foragers as test ants, then repeated with nurses as test ants. A solvent control of clean diethyl ether was tested on 10 foragers and 10 nurses. Overall, 12 nurse extractions were tested on 22 foragers and 25 nurses and 12 forager extractions were tested on 23 foragers and 27 nurses. All random numbers were generated using the RANDBETWEEN() function in Microsoft excel.

3.3.5 Concentrated hindgut extraction and derivitisation for GCMS analysis

A more concentrated sample was required for chemical analysis to ensure that the trail pheromone and any other detectable compounds were above the limits of detection of the GCMS instrument. To achieve this, 15 hindguts were dissected in air and placed into a vial containing 80µl diethyl ether with mellein as an internal standard at a concentration of 0.75ng/µl. This was repeated for each test colony producing 6 extractions of forager hindguts and 6 extractions of nurse hindguts. The vials were then placed into a sonicator bath (5 minutes) and a vortex mixer (1 minute). The liquid phase was then transferred to a clean vial and gently blown down to dryness under a gentle stream of oxygen free nitrogen (OFN). The samples were resuspended in 15µl of toluene and 2µl were injected onto the GCMS instrument. After the first injection was performed the samples were silylated to allow analysis of any compounds which were too polar for analysis using the non-polar GCMS column used here (detailed below). A

solution of 4-n-nonylphenol in toluene was made at a concentration of 100ng/µl. 0.6µl of this solution (60ng of nonylphenol) was added to each sample as a second internal standard which was also used to confirm that the silylation reaction took place (noting that it was correctly converted to the trimethylsilyl (TMS) ether). The samples were then blown down to dryness under a stream of OFN, and 5µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and 10µl pyridine were added. To facilitate the derivitisation reaction, the samples were heated to 60°C for half an hour. 2µl of each sample were injected onto the GCMS instrument.

3.3.6 GC-MS analysis and semi-quantitation of trail pheromone

GCMS was performed on a Thermo Trace GC Ultra linked to a Thermo ITQ 1100 mass spectrometer. The injection was splitless, and helium was used as a carrier gas at a flow rate of 1.3ml/min. The column used was an Agilent DB-5ms Ultra Inert, 30m x 0.25mm, 0.25 µm film. The GC oven was set to the following temperature programme; hold at 60°C for 4 minutes; ramp to 300°C at 10°C/min; hold at 300°C for 2 minutes. The mass spectrometer was used in EI mode (70eV), the scanning range was between m/z 40 and 650. Chromatograms were analyzed using Thermo Xcalibur software (Thermo Xcalibur 2.3 build 26) and compounds were tentatively identified by comparison with the NIST/Wiley mass spectral database (NIST MS Search 2.0 g). When tentative identifications were made, mass spectra and retention times were compared with synthetic chemical standards to attempt to confirm the identifications. The relative abundance of trail pheromone of nurses and foragers was calculated for each sample by integrating the area under the trail pheromone peak on the total ion chromatogram and dividing by the area under the peak for the mellein internal

standard. This gave trail pheromone abundance in ng mellein equivalents. To ensure analytical consistency each sample was injected twice; consistency was determined by including data from both replicates in the initial multivariate analysis. In all cases both replicates clustered very closely to each other so analytical consistency was confirmed. For subsequent statistical analysis, one of the two repeat injections for each sample was randomly selected and removed from the dataset.

3.3.7 Statistical analyses

The circle bioassay data was analysed using generalised linear models (GLMMs) with Poisson distributions and log link functions. Donor colony and test colony were included as random factors in the models to account for the repeated testing of glandular extracts taken from the same colonies, and the repeated use of test colonies as a source of test ants. Non-significant interaction terms were eliminated stepwise from models containing all explanatory variables until minimum adequate models were produced. The minimum adequate model analysed how the number of arcs crossed by test ants was affected by the donor caste, the donor colony and the test colony.

The trail pheromone abundance data were analysed using Mann-Whitney U tests which were performed in R version 3.1.0 (R Core Team, 2014). To correct for multiple comparisons (multiple compounds were tested for a statistically significant difference in concentration between nurse and forager extractions), p-values were calculated using the Bonferroni Correction.

To perform multivariate analysis of other compounds, the raw GCMS data were first processed using SIEVE software. The chromatograms were automatically aligned then binned using 1 Da mass and 0.3 minute retention time windows. All signal intensities were normalized to a mellein internal standard signal (m/z 134 at RT 15.98). The non-derivatized signal intensities were multiplied by a factor of 1000 and the derivatized signal intensities were multiplied by 100,000 to ensure that no values were less than 1 to allow for a subsequent log transformation. Datasets were then transferred to SIMCA 14 software (14.0.0, build 1359, Umetrics) for multivariate analysis. First of all, principal component analyses (PCAs) were performed separately for the derivatized and non-derivatized datasets to obtain an overview of the data and detect any outliers. In order to ascertain which GCMS signals were contributing most to the variation between classes (nurses vs. foragers) orthoganol partial least squares discriminant (OPLS-DA) analyses were used. The performance of these models was assessed by examining the amount of variation that they explained (R^2X for PCA and R^2Y for OPLS-DA) and their predictive power (Q^2X or Q^2Y). This approach produces an S-plot for each model, which are scatter plots of the loading profiles generated by OPLS-DA that display covariance and correlation values. Discriminatory signals were identified by examining these S-plots. After identifying discriminatory signals, their retention time and m/z were compared with the original sample mass chromatograms to identify the discriminatory compound. The areas of the peaks of discriminatory compounds relative to the mellein internal standard were then compared between nurses and foragers. The relative abundance of the compound in extracts from the two castes was analysed using a Mann-Whitney U test as the data were not distributed normally.

3.4 Results

3.4.1 Trail following bioassay to test potency of nurse and forager hindguts

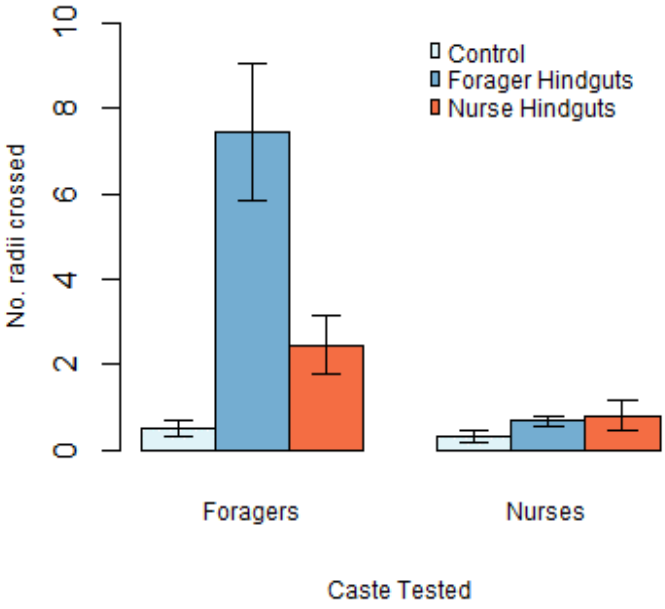

Fig 3. – Foragers followed extractions of forager hindguts more so than nurse hindguts. The bars show the mean number of bioassay radii crossed by nurse and forager test ants when tested with a solvent control (light blue), forager hindgut extract (blue) and nurse hindgut (orange) extract. The error bars display standard error.

Extractions were made from either forager hindguts or nurse hindguts, and tested for potency on a trail following bioassay using both nurse and forager test ants; the assay results are shown in Figure 3. The data show that when foragers were tested they crossed significantly more radii when following a trail consisting of forager hindgut extract than a nurse hindgut extract *($\beta = 1.1937$, $SE = 0.1595$, $z = 7.5$, $p < 0.001$)* or a solvent control *($\beta = 2.3839$, $SE = 1.0375$, $z = 2.298$, $p = 0.0216$).* Test foragers also followed an extraction of nurse hindguts significantly further than they did the solvent control (*$\beta = 1.6539$, $SE = 0.5847$, $z = 2.828$, $p = 0.0047$).* When nurses were tested on either extract type, their response was not significantly different than to a solvent control (nurses tested on nurse hindguts; *$\beta = 0.7805$, $SE = 0.8690$, $z = 0.898$, $p = 0.3691$*; nurses tested on forager hindguts; *$\beta = 0.7985$, $SE = 0.6239$, $z = 1.280$, $p = 0.2$*).

3.4.2 Semi-quantitation of trail pheromone in nurse and forager hindguts

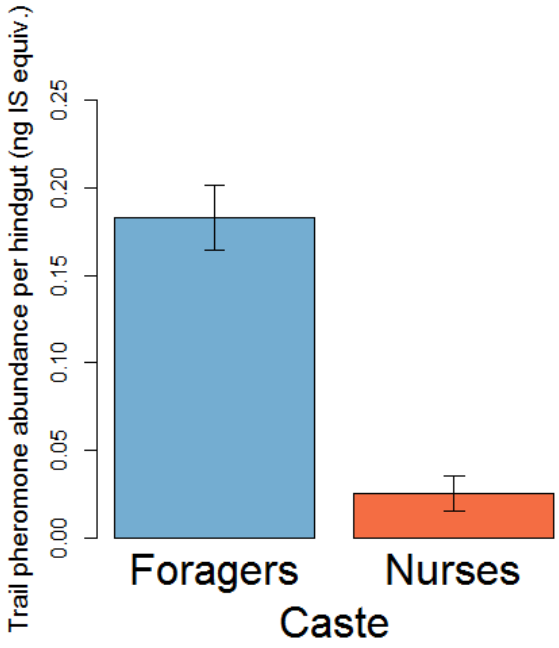

Fig 4. – The relative abundance of trail pheromone to a mellein internal standard. The bars show the mean relative abundance from six composite extractions of 15 hindguts for each caste and the error bars show the standard error.

To investigate the bioassay result further, that test foragers followed an extraction of forager hindguts farther than an extraction of nurse hindguts, more concentrated extracts were made and analysed using GCMS and the amount of trail pheromone was semi-quantified. Trail pheromone (3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin) was semi-quantified by calculating the abundance relative to the analogous compound mellein which was included as an internal standard in six repeated extractions of nurse and forager hindguts. The results are shown in Figure 4. The mean relative abundance of trail pheromone in a forager hindgut was calculated to be 0.18ng mellein equivalents, whereas nurse hindguts contained an average relative abundance of just 0.028ng mellein equivalents, a 6-fold difference. This difference was statistically significant (*p = 0.002, W = 36).*

3.4.3 Multivariate analysis of compounds found in the hindguts of nurses and foragers

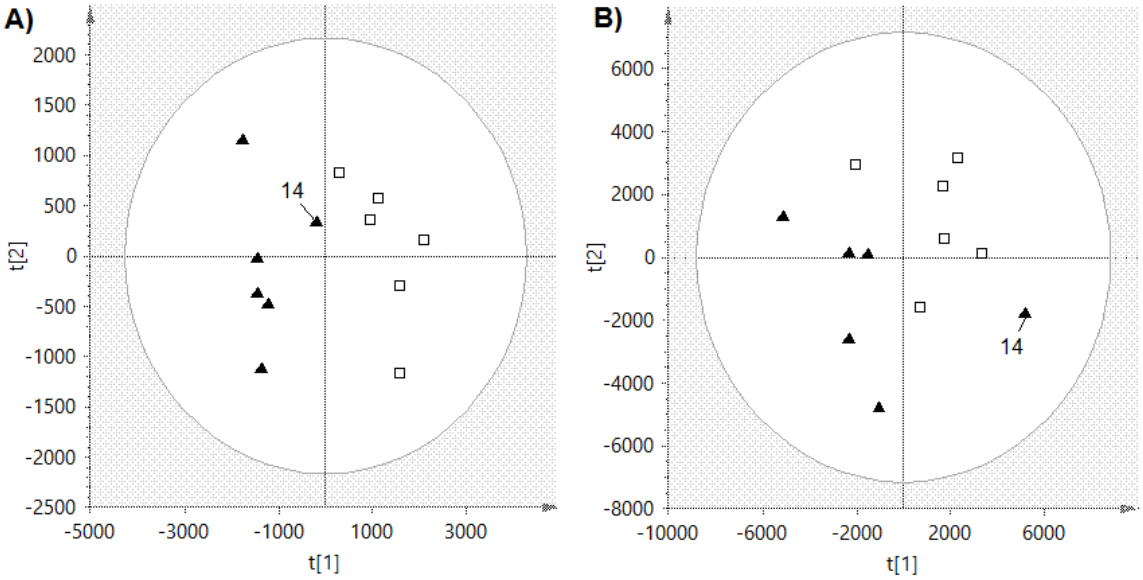

Fig. 5 – PCA scores plots of the profiles of GCMS signal abundances in extractions of hindguts from nurse ants (black triangles) and forager ants (white squares)**.** Composite samples were created by dissecting glands from 15 workers for each caste; this was repeated for six colonies. **A)** displays non-derivatized data whereas **B)** displays the data from extractions after a trimethylsilyl derivatization reaction. Although there were no true outliers, the data for nurses from colony 14 does not cluster with the rest of the nurse samples in the derivatised data.

Multivariate statistical analysis was used to determine whether the pattern of variation of compounds found in the concentrated hindgut extractions differed between nurse and forager castes. The analyses were performed separately for the derivatised and non-derivatised datasets. The PCAs for both the derivatized and non-derivatized datasets showed that there was clustering based on caste present in the data, although there was some overlap in the derivatized data (see fig. 5). There were no outliers present so no data were excluded from the analysis, however nurses from colony 14 did not cluster with other nurse samples in the derivatised data. PCA does not take class (in this case the class was worker caste) into account so supervised OPLS-DA models were also created for the two datasets. These models increased the explanatory power and predictive ability of the models (see Table 1), particularly in the derivatised dataset, where R2Y increased from 0.562 to 0.96 and Q2Y increased from 0.326 to 0.688.

Dataset	Multivariate method	Components	R^2X / R^2Y	Q^2X / Q^2Y
Non-Derivatised	PCA	2	0.763	0.617
Non-Derivatised	OPLS-DA	1+1+0	0.936	0.834
Derivatised	PCA	2	0.562	0.326
Derivatised	OPLS-DA	1+2+0	0.96	0.688

Table 1 – Performance parameters of multivariate discriminate models for the comparison of GCMS signal abundances from nurse ant and forager ant hindgut extractions.

The visual output of the OPLS-DA models is shown in Figure 6. The data show clear clustering based on whether extractions were made from forager or nurse hindguts and the non-derivatized data separated well with just a single component. This clustering combined with the high quality model diagnostics described above indicate that compounds were present in the dataset which were discriminatory markers of worker caste. To further investigate these discriminatory compounds the S-plots were investigated, which show the GCMS-signals that contribute most to the separation between worker castes. The S-plots are shown in Figure 7. Both of the plots for the non-derivatized and derivatized data are slightly unusual in that there were few signals

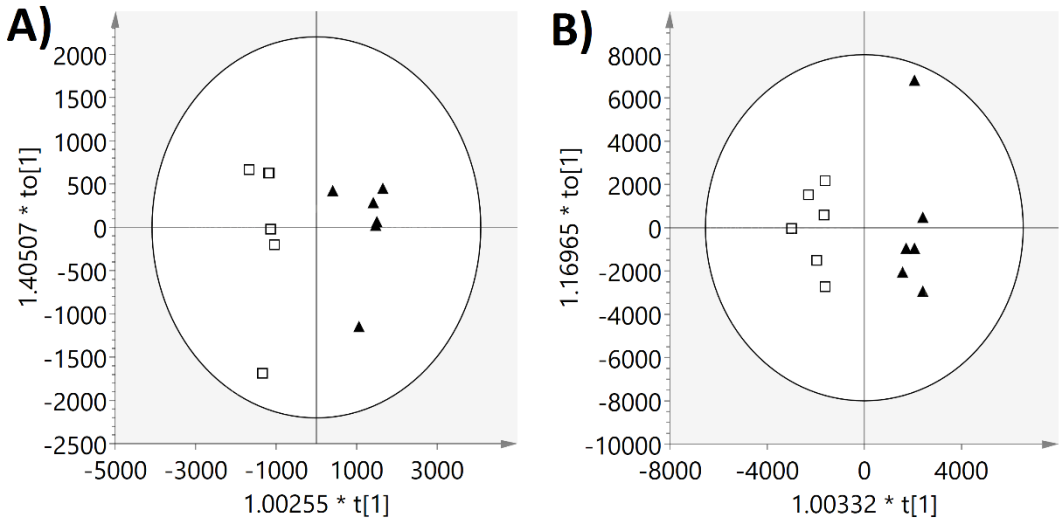

Fig. 6 – OPLS-DA scores plots of the profiles of GCMS signal abundances in extractions of hindguts from nurse ants (black triangles) and forager ants (white squares). Composite samples were created by dissecting glands from 15 workers for each caste; this was repeated for six colonies. **A)** displays non-derivatized data whereas **B)** displays the data from extractions after a trimethylsilyl derivatization reaction.

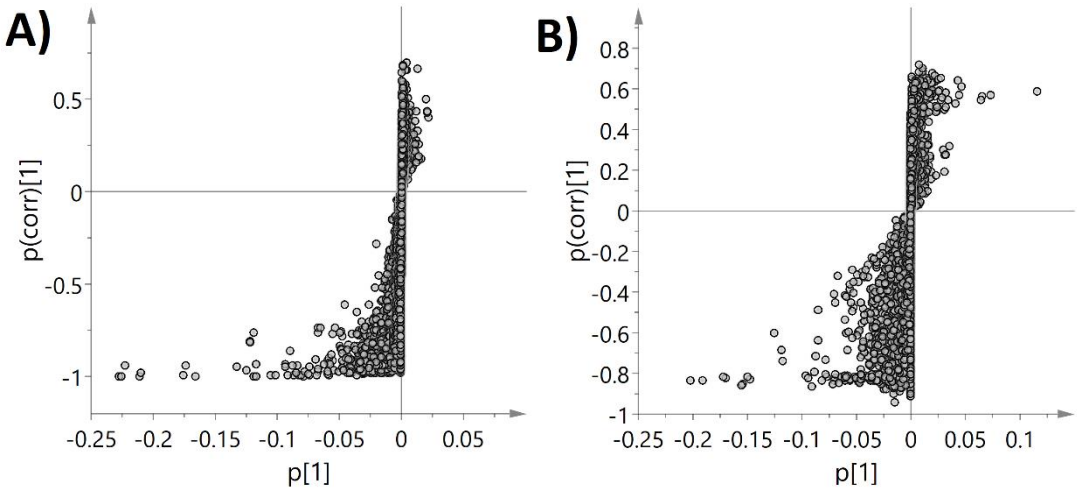

Fig. 7 – S-plots for the OPLS-DA models showing how GCMS signals differed between extracts from nurses and foragers. Each circle represents a single fragment of a compound detected at a specific retention time. A single compound is represented by multiple data points (fragments) on these graphs. Signals in the bottom left of the plots were more abundant in foragers, while those in the top right were more abundant in nurses. **A)** displays the non-derivatized data, **B)** displays the derivatized data.

that were stronger in the nurse extractions; this is especially evident in the non-derivatized data. This may represent an overall upregulation of compound production in foragers. However there were some signals in the derivatized data that were more abundant in the nurse extractions.

Discriminatory signals were scrutinised by selecting signals from the bottom-left and top-right of the data in the S-plots, and variable trend plots were created to ascertain whether signals did appear to vary based on the caste of ant used in the extractions. An example variable trend plot is shown in Figure 8. Subsequently, Mann-Whitney U tests (with appropriate Bonferroni corrections) were used to analyse differences in the abundance of compounds relative to the mellein standard, significant differences were found for 18 compounds, shown in Table 2. Four compounds were found to be more abundant in nurses than foragers. All of these compounds appear to be amino acids except one (N4) which could not be identified by comparison with databases. 14 compounds were found to be more abundant in foragers, one of these compounds

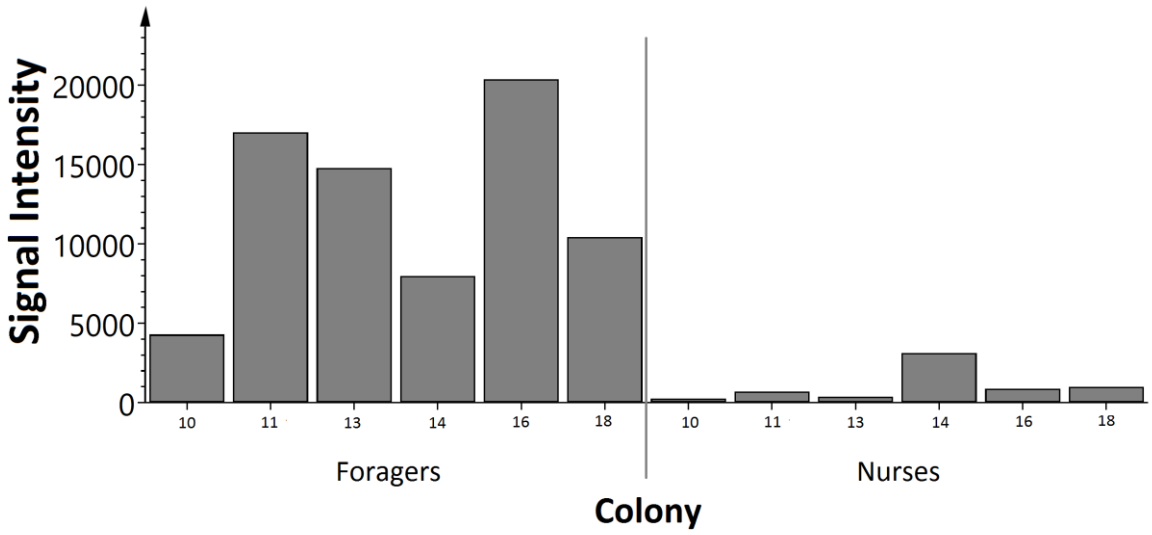

Fig. 8 – The variable trend plot for a single data point from the S-plot shown in figure 6A which clearly shows signal intensities that were stronger in extractions of forager hindguts than nurse hindguts. This particular signal is from a fragment of 41 m/z at retention time 22.88 in the non-derivatized data. This fragment corresponds with compound F7 in Table 2 which was tentatively assigned the class of a carboxylic acid ester. Each number on the x-axis represent a test colony.

was the trail pheromone of *L. niger* (F3 – 3,4-dihydro-8-hydroxytrimethylisocoumarin). The other compounds were a mixture of hydrocarbons (F6, F12 and F14), esters (F7-F11 and F13), a carboxylic acid (F1), a terpene (F4) and a sesquiterpene (F5). Database searches for compounds with a spectra matching that of compounds F5 produced two compounds with the same mass spectrum: elemene and germacrene, both sesquiterpenes. In other cases more than two database spectra matched those of the chromatographic peak (F6, F7, F9, F11 and F13); here the most likely class of the compound was recorded. The mass spectra of a series of synthetic alkane standards was compared with those of the tentatively identified alkanes (F12 and F14) to attempt to confirm the tentative identifications. The mass spectra and retention time of F12 matched that of heptacosane, while F14 matched that of nonacosane. Comparisons between the spectra of chromatographic peaks from the extractions and those of the NIST/MS databases are shown in Appendix III at the end of this thesis.

#	More abundant in	Derivatized?	Retention Time (Minutes)	Ions	ID	Mean Relative Abundance in Foragers (8ng mellein equivalent)	Mean Relative Abundance in Nurses (8ng mellein equivalent)	Fold Difference	p (adjusted)	Kovats Index
N1	Nurses	Yes	8.72	73, 102, 147, 204	Glycine (TMS ESTER)	0.005 ± 0.003	0.018 ± 0.010	3.76	0.024	1066
N2	Nurses	Yes	9.98	45, 59, 73, 102, 115, 131, 147, 176, 204	Glycine (TMS ESTER)	23.94 ± 7.24	29.22 ± 11.44	1.22	0.027	1138
N3	Nurses	Yes	12.3	45, 73, 86, 100, 133, 147, 158, 170, 203, 218, 232, 260	Isoleucine/Leucine (TMS ESTER)	4.30 ± 2.05	8.79 ± 3.26	2.04	0.045	1287
N4	Nurses	No	15.42	78, 104	???	0.0008 ± 0.0004	0.0071 ± 0.0073	9.27	0.008	1521
F1	Foragers	Yes	10.4	45, 59, 66, 73, 115, 131, 133, 147, 233	Hydroxymethylpropanoic acid (bis-TMS Ester)	0.90 ± 0.51	0.37 ± 0.07	2.43	0.018	1164
F2	Foragers	Yes	13.67	158	???	0.11 ± 0.10	0.04 ± 0.03	3.02	0.13	1386
F3	Foragers	No	18.5	91, 115, 134, 145, 162, 173, 188, 206	3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin	0.18 ± 0.085	0.028 ± 0.021	6.43	0.048	1789
F4	Foragers	No	18.89	43, 53, 67, 81, 91, 95, 109	Methyloctadiene	0.45 ± 0.11	0.06 ± 0.05	8.04	0.024	1826
F5	Foragers	No	19.12	41, 67, 79, 91, 105, 121, 133, 147, 161, 175, 189, 204	Elemene/Germacrene	3.58 ± 1.21	0.46 ± 0.44	7.78	0.024	1848
F6	Foragers	No	20.9	43, 55, 67, 81, 95, 109, 123, 137, 165	Alkyne	1.89 ± 1.54	0.07 ± 0.10	25.91	0.048	2025
F7	Foragers	No	22.88	41, 55, 67, 81, 101, 111, 123, 139, 159, 183, 213, 149, 269	Carboxylic acid ester?	1.28 ± 1.26	0.03 ± 0.04	48.26	0.048	2243
F8	Foragers	No	24.12	41, 55, 67, 81, 101, 111, 129, 143, 155, 173, 201, 213, 297	Decanoic acid, decyl ester	5.39 ± 3.32	0.19 ± 0.21	28.30	0.024	2389
F9	Foragers	No	25	41, 55, 67, 83, 95, 109, 125, 143, 153, 171, 193, 201, 213, 221, 241, 269	Carboxylic acid ester?	0.41 ± 0.37	0.01 ± 0.02	45.03	0.048	2498
F10	Foragers	No	25.7	41, 55, 67, 83, 97, 111, 129, 143, 157, 171, 183, 201, 213, 256	Butyl laurate	17.56 ± 9.12	1.26 ± 1.50	13.94	0.048	2588
F11	Foragers	No	26.26	41, 55, 67, 83, 97, 111, 129, 140, 149, 157, 171, 177, 185, 215, 256	Propyl dodecanoate	7.08 ± 4.43	0.59 ± 0.75	12.01	0.048	2663
F12	Foragers	No	26.7	41, 57, 71, 85, 99, 111, 127, 141, 155, 169, 183, 197, 225, 239	Heptacosane*	0.77 ± 0.32	0.17 ± 0.14	4.42	0.024	2722
F13	Foragers	No	27.6	41, 55, 67, 81, 101, 111, 123, 141, 159, 297, 353	Carboxylic acid ester?	0.26 ± 0.14	0.02 ± 0.02	17.05	0.048	2849
F14	Foragers	No	28.13	41, 57, 71, 85, 99, 112, 127, 141, 155, 169, 183, 193, 211, 225, 239, 253, 267	Nonacosane*	0.73 ± 0.30	0.16 ± 0.12	4.44	0.024	2935

Table 2 – Compounds contributing most to the chemical differentiation of nurse and forager hindguts as found by OPLS-DA models. All abundances are relative to the mellein internal standard and are the mean of six composite samples, each taken from a separate colony and containing 15 hindguts from either nurses or foragers. The p values were calculated using Mann-Whitney U tests and were adjusted using a Bonferroni correction. Compounds were tentatively identified by comparing their mass spectra to databases of known spectra. The ID's followed by a '?' indicate only the class of the compound could be inferred from comparisons with the databases. Those followed by a '*' indicate identifications confirmed by matching retention times and mass spectra with those of synthetic standards.

3.5 Discussion

The response of test ants to hindgut extractions from nurses and foragers showed a marked difference when they were tested in the circle assay. It is clear that when foragers were used as test ants, forager hindguts were far more potent than those of nurses, although nurse hindguts were still followed for a short distance. This result indicated two things; that more trail pheromone was present in forager hindguts than in nurse hindguts, or forager hindguts contained a different chemical, or blend of chemicals, that was more attractive to foragers than to nurses. It is interesting to note that nurses themselves did not follow trails of either nurse or forager hindguts significantly further than they did a solvent control. This confirms that nurses as a temporal caste cannot take part in foraging behaviour. The physiology underlying this observation is not currently understood, perhaps nurses have not yet developed the appropriate receptors to detect trail pheromone. Castillo and Pietrantonio (2013) found that the brains of different subcastes of *Solenopsis invicta* workers contained constant and differential patterns of short neuropeptide F receptors. These receptors appear to be involved in nutrient sensing and/or brood care and are located in the mushroom bodies, central complex, lateral horn and antennal lobes of the brain and therefore were theorised to play a role in behavioural polyethism between subcastes. Another potential explanation is that the trail pheromone following response is not innate, and that worker ants may need to learn to follow trail pheromone. Cammaerts (2013) found that young *Myrmica rubra* workers (which are more likely to be nurses) were not capable of following a pheromone trail efficiently. They became more

proficient when older, experienced workers were also following the trail, and eventually became proficient themselves.

The GCMS analysis of trail pheromone abundance confirms the result that there was more trail pheromone present in the hindguts of foragers than there was in the hindguts of nurses. The abundance relative to the mellein standard was over 6 times higher in the forager hindguts than in the nurse hindguts. The behavioural result of the circle assay discussed above also confirms that this difference in abundance is significant at a behavioural level; foragers followed trails of forager hindguts almost 4 times further than they did trails of nurse hindguts. This supports the idea that the chemical toolkit of *L. niger* is customized to suit the tasks performed by the temporal caste. It is, however, just a single compound in an organism that produces potentially hundreds of different pheromones.

The multivariate analysis of chemicals found in the hindgut extractions identified distinct patterns present in the variation of those chemicals. These patterns were dependant on the behavioural caste the extractions came from. S-plots from OPLS-DA models showed which compounds were contributing most to the differences in the pattern of variation between the two castes. Three of the four compounds found in a higher abundance in nurse hindguts were amino acids (N1, N2 and N3 in Table 2), although they were only found at relative abundances 1.2-3.8 times higher than the levels found in forager hindguts. This could be explained by nurses storing proteinaceous food to feed to the growing larvae or to the queen. The majority of protein and amino acids gathered by ant colonies is fed to larvae to promote growth, and to the queen to fuel egg production (Sorenşen et al., 1981). Ant workers store

communal food in their crops, which is a part of their digestive system. It is likely that traces of food in the crop can make their way further down the digestive system to the hindgut. If proteinaceous food is stored for longer in nurse crops than forager crops, that may explain why more makes its way down the digestive tract in nurses than in foragers. Alternatively, this could be explained by nurses requiring amino acids to produce trophic eggs. Trophic eggs differ in shape to reproductive eggs, and sometimes completely lack detectable DNA. They are produced by some species of ant purely to feed to the larvae or the queen, and this behaviour has been observed in the closely related species *Formica rufa* (Hölldobler and Wilson, 1990).

The compounds which were more abundant in foragers than in nurses were a mixture of classes. There were carboxylic acids and esters (F1, F7-F11 and F13), a terpene and a sesquiterpene (F4 and F5), two alkanes (F12 and F13), an alkyne (F6) and the trail pheromone, a dihydroisocoumarin (F3). The function of 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin is well established as the trail pheromone of *L. niger* (Bestmann et al., 1992). As their name suggests, foragers perform foraging for the colony, so it is logical that their hindguts contain more trail pheromone than nurses which do not forage. This means that nurses do not expend energy synthesizing trail pheromone which they will not use. The presence of a carboxylic acid is difficult to assign a function to; as discussed in Chapter 2 the function of these compounds in formicine ants has yet to be investigated. Some of the other classes of compounds in the forager toolkit have been found to have repellent or defensive properties. Esters and alkanes are commonly theorized to be wetting agents used by arthropods to assist in the spread of other defensive chemicals such as formic acid (Blum, 1996; Francke et al.,

2014), so these compounds may be used by *L. niger* to defend the nest against other ants and insects. In addition to foraging, foragers also take part in nest defence for which these compounds may play a role. Again, it is logical that nurses do not expend energy synthesizing defensive compounds when they do not participate in nest defence. Two potential identifications were found for the sesquiterpene (F5) with matching mass spectra (see Appendix III), suggesting the structure of elemene or germacrene. Elemene is a compound more frequently associated with plants than insects (Ibrahim et al., 2008; Zoghbi et al., 1995), although it was identified in the venom gland secretion of *Solenopsis geminata* ant queens (Cruz-López et al., 2001) but its function was not investigated. Germacrene is one of a number of different chemicals used by the black bean aphid, *Aphis fabae*, to locate its host plant, *Vicia faba* and electro-antennograms showed that aphid antennae were particularly sensitive to this compound (Webster et al., 2008). *L. niger* are known to feed from *A. fabae* honeydew and have been observed to deter aphid predators (Banks, 1962). Perhaps *L. niger* use germacrene to attract aphids, or prevent them from fleeing when approached. Alternatively the ants may sequester the compound from the honeydew that they consume, although this study was performed on lab colonies that had no access to aphids for at least a week.

It appears that chemicals which were significantly more abundant in forager hindgut extractions are probably used in defence and foraging; both are tasks performed by the forager subcaste. On the other hand, those chemicals more abundant in nurse hindgut extractions appear not to be produced by nurses, but are more likely present as a consequence of performing nurse-related tasks such as feeding brood. It is clear

that there are differences in the chemicals present in hindgut extractions of nurse and forager workers of *L. niger,* and that the chemical toolkit of the forager subcaste is suited to the tasks they perform.

4 Identification of two recruitment pheromones of *Lasius flavus*

4.1 Abstract

The work presented in Chapter 2 of this thesis investigated the contents of the hindgut of *Lasius flavus*, a gland known to contain trail pheromone. Unfortunately no chemicals were detected in this gland, and the identity of the trail pheromone remained a mystery. In this chapter further work was performed using a combination of liquid chemical extraction, high performance liquid chromatography and sensitive bioassays to identify recruitment pheromones in *L. flavus.* Two recruitment chemicals were identified and their structures elucidated using both GCMS and comparison with chemical standards. One identified pheromone was 2,6-dimethyl-5-heptenol (DMH) and the second was mellein. The source of DMH was found to be the heads of worker ants, while mellein was found to originate in the hindgut. Further comparative bioassays were used to elucidate the behavioural function of the two recruitment pheromones. DMH was found to cause a heightened level of aggression in worker ants, indicating that its probable function is that of an alarm pheromone. Mellein was found to be effective at eliciting trail following behaviour and is therefore likely to be the trail pheromone of *L. flavus*. Mellein was found at a concentration of just 5.56pg per hindgut, a concentration ten-fold lower than the congeneric species *L. fuliginosus*, and thirty-fold lower than *L. niger.* This is also the lowest concentration of pheromone ever detected in an ant. Workers were also highly sensitive to its presence and followed a trail of just 0.01pg per cm; to date this is the lowest known detectable concentration

of a pheromone by an ant. The work presented here lays a strong foundation for the further study of low-concentration pheromones of other species and highlights the need to study putative pheromones in a comparative manner.

4.2 Introduction

Communication between conspecifics is widespread in the animal kingdom. Communicated information can allow individuals to share food with conspecifics, or alert other individuals to the presence of predators. The signals used to communicate are varied and cover a range of sensory modalities; gestures can be visual or tactile, vocal signals are audial and pheromones (or chemicals) are olfactory. The social insects are capable of encoding complex information in complex communication signals. For example, honey bees use a waggle dance to convey the distance and direction of food sources to the rest of the colony (von Frisch, 1967), and many ant species use mixtures of pheromones to communicate a huge variety of messages to their nestmates (Hölldobler and Wilson, 1990). Ants provide an ideal model for the study of pheromonal communication systems, as each species can produce a myriad of chemical signals, and their terrestrial lifestyle means their behaviours are easy to observe. A subset of the pheromones used by insects are known as recruitment pheromones. Their purpose is to attract conspecifics to the source of the pheromone. There are two major uses of recruitment pheromones in the ants; to guide nestmates to a food source or nest site by laying trails of an attractive pheromone (a trail pheromone), and to attract nestmates to a threat (for example, a predatory intruder)

by exuding an alarm pheromone into the air. This allows nestmates to assist the alarmed ant in dealing with the threat.

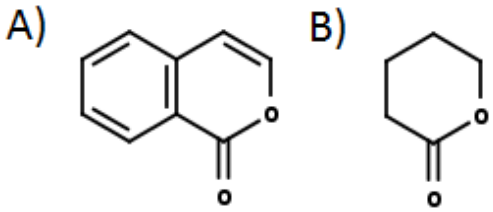

Fig. 1 – The two basic structures of all formicine ant pheromones discovered to date. **A)** Isocoumarin **B)** δ-lactone

Previous work has identified the trail pheromones of over 60 species of ant, 16 of which are from the subfamily formicinae (Morgan, 2009). The trail pheromones of formicine ants have been particularly difficult to identify due to the relatively minute amounts of trail pheromone that they use; many myrmicine and ponerine ant glands contain 1-100ng of trail pheromone, whereas in the formicine ants this value tends to be less than 150pg (Morgan, 2009). In all cases where a formicine species' trail pheromone has been identified, it has been located in the hindgut; a small gland which forms the final part of the digestive tract and exits the gaster of the ant via the acidopore. All identified formicine trail pheromones are based on one of two potential chemical structures; either an isocoumarin structure (Figure 1A) or a δ-lactone structure (Figure 1B) (Morgan, 2009). Of the 16 formicine ants with identified trail pheromones only two are in the genus *Lasius*, despite this genus being hugely ecologically important within Europe (Boots and Clipson, 2013; Czechowski et al., 2013b; Vlasáková and Raabová, 2009). In *Lasius niger* the trail pheromone was identified to be 3,4-dihydro-8-hydroxy-

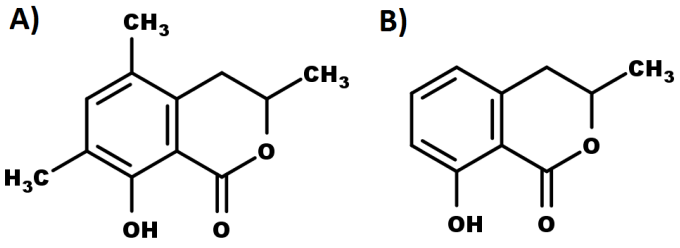

Fig.2 – The only two trail pheromones identified from *Lasius* ants. A) 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin was discovered in *L. niger* hinguts. B) Mellein was discovered in *L. fuliginosus* hindguts.

3,5,7-trimethylisocoumarin (Bestmann *et al*., 1992), and in *Lasius fuliginosus* it was 3,4-dihydro-8-hydroxy-3-methylisocoumarin, or mellein (Kern *et al*., 1997) The structures of these compounds are shown in Figure 2.

Alarm pheromones have been more widely studied than trail pheromones. This is primarily because they are present in more species (even non eusocial species can use alarm pheromones), but also because they are usually present in larger quantities than other pheromones (Blum, 1969). Alarm pheromones in ants are often multicomponent and are produced by and stored in a suite of glands. In formicine ants, alarm pheromones are generally stored and released from the mandibular gland, poison gland and Dufour gland (Hölldobler and Wilson, 1990). Alarm compounds in the mandibular gland most commonly have terpenoid structures such as citronellal; the poison gland often contains large amounts of formic acid; and the Dufour gland contains long chain hydrocarbons, most commonly undecane. Terpenoid compounds from mandibular glands often attract worker ants to the source of the pheromone (Hölldobler and Wilson, 1990), and Ayre and Blum (1971) found that formic acid and undecane caused a strong attraction response in *Camponotus pennsylvanicus* worker ants. In the genus *Lasius,* six compound have been described as alarm pheromones from six different species. These compounds are summarised in Table 1.

The studies on alarm pheromones described above performed behavioural work to demonstrate the attractive effect of these compounds. However, the response of an ant to alarm pheromone is not simply just attraction (Hölldobler and Wilson, 1990). The other effects, such as increased agitation and tendency to aggress can be difficult to quantify. One technique has been developed known as the mandible opening

Compound	Gland	Species	Reference
Undecane	Dufour gland	*L. alienus, L. fuliginosus, L. niger*	Regnier (1969); Dumpert (1972); Bergstrom (1970)
Citronellal	Mandibular gland	*L. spatheus, L. umbratus, L. claviger*	Regnier (1968)
2,6-dimethyl-hepten-1-al	Mandibular gland	*L. claviger*	Regnier (1968)
2,6-dimethyl-hepten-1-ol	Mandibular gland	*L. claviger*	Regnier (1968)
Citronellol	Mandibular gland	*L. claviger*	Regnier (1968)
Nerol	Mandibular gland	*L. claviger*	Regnier (1968)
Geraniol	Mandibular gland	*L. claviger*	Regnier (1968)
Tridecane	Dufour gland	*L. claviger*	Regnier (1968)

Table 1 – Compounds from ant species in the genus *Lasius* which have been described as alarm pheromones.

response assay which quantifies increased aggression levels by measuring the proportion of a sample of harnessed ants, which exhibit a threatening, gaping mandible posture when presented with a range of stimuli (Guerrieri and d'Ettorre, 2008). This assay was developed when testing the aggression response of workers to nestmate and non-nestmate cuticular hydrocarbons, but should also be applicable to the testing of alarm pheromones. The studies described so far in this chapter have not tested anything beyond the attractive response of workers to alarm pheromone, so it is unclear how many of the described compounds are actually used by ants as alarm pheromones. Furthermore, as both trail pheromones and alarm pheromones are known to cause attraction in worker ants, it is important to compare and contrast the responses of workers to both types of pheromone in bioassays designed to assess more specific behaviour. This is not currently routinely done by studies in this field and as a result conclusions are often unclear. For this reason, it is important that ant responses are studied comparatively, by using robust and relevant bioassays to confirm the proposed biological function of any identified pheromones.

One member of the *Lasius* genus which has so far been relatively neglected by the chemical ecology research community is the yellow meadow ant, *Lasius flavus.* This

species of ant is ubiquitous throughout Northern Europe, and is the dominant ant species in meadow grassland (Czechowski et al., 2013b). It is an ecosystem engineer which accelerates primary succession of grassland into forest (Vlasáková and Raabová, 2009) and affects the physicochemical properties of the soil they inhabit (Boots and Clipson, 2013; Wu *et al*., 2015). An unusual life history trait of *L. flavus* is that the workers forage almost entirely underground, not on the surface like other congeneric species such as *Lasius niger.* This subterranean lifestyle means that foraging workers are exposed to a much more restricted air flow than surface foraging species. Along with the generally cooler temperatures found underground, this may mean that any chemicals deposited by the ants are likely to evaporate much more slowly from the environment. As a result, it is possible that pheromones produced by this species are present in much lower quantities compared to species that forage primarily above ground.

Here, I use a novel combination of high resolution chromatography, biologically relevant bioassays and sensitive analytical chemistry to both identify recruitment pheromones of *L. flavus*, and use robust and relevant bioassays to compare the specific behavioural effects of worker ants when exposed to alarm and trail pheromones. Previous work has shown that all trail pheromones discovered to date in formicine ants have been present in the hindgut (Morgan, 2009). In addition, solvent extractions of *Lasius flavus* hindguts elicited a trail following response in test workers (Jones, 2014) while Dufour gland and poison gland extractions did not, thus indicating that the trail pheromone of *L. flavus* is also in the hindgut. The amount of trail pheromone present in *L fuliginosus* hindguts was just 50-100pg (Kern et al., 1997), and

work presented in Chapter 3 of this thesis found an average of 180pg in the hindguts of *L. niger* foragers. The work performed in Chapter 2 of this thesis revealed that the only compounds present in liquid extractions of *L. flavus* hindguts were also found in other glands in higher quantities, indicating that they were most likely the result of cross contamination. This indicated that, in order to further explore *Lasius* trail pheromones, improved analytical methods need to be developed. These methods must be able to detect substances that elicit trail following behaviour and may only be present in sub-nanogram quantities.

Based on previous studies on the *Lasius* genus, and that *L. flavus* has a predominantly subterranean life history. I concluded that the trail pheromone (or pheromones) is present at sub-nanogram quantities and probably possesses an isocoumarin-like structure. Based on previous studies of *Lasius* alarm pheromones, I predicted that the alarm pheromone would elicit some level of attraction from worker ants and likely possesses a terpenoid structure. When comparing the behavioural responses to alarm and trail pheromones, I expected that alarm pheromones will cause a heightened aggression response, while trail pheromones would elicit a stronger trail following response

4.3 Materials and methods

4.3.1 Chemicals

All solvents were HPLC grade and were purchased from Rathburn Chemicals Ltd, UK. Mellein was purchased from Cayman Chemical and all other chemicals were purchased from Sigma Aldrich, UK.

4.3.2 Collection and storage of ants

Eight *Lasius flavus* colonies were collected from the University of Sussex campus (Falmer, Brighton, UK). Colonies were located by searching for the tell-tale mounds formed by *L. flavus* in undisturbed meadow grassland. Once a mound was located, the colony was dug out of the ground ensuring workers and brood were collected along with the soil. The queenless colony fragments and soil were then housed in plastic tubs (30 cm x 45 cm x 25 cm) and the walls were coated in fluon to prevent escape. Each fragment contained approximately 1000-3000 workers and brood at various stages of development (eggs, larvae and pupae). The colonies were kept in a natural day-night cycle, and the temperature varied between 20°C and 24°C. When not being used assays the colonies were fed twice weekly on mealworms (*Tenebrio molitor* larvae) and a 1:2 honey:water mixture. Water was provided ad libitum. The colonies were kept in the lab for 2-3 weeks before being used in experiments.

4.3.3 Extraction of pheromones from ant workers

Approximately 5300 *L. flavus* workers taken from six lab colonies were collected for pheromone extraction. Previous work has revealed that the hindguts of foraging *Lasius*

niger workers contain more trail pheromone than nurses which remain inside the nest (see Chapter 3). Therefore in order to maximise the amount of trail pheromone collected, a foraging trail was set up to drops of 1M sucrose solution from each of the six colonies and ants were only collected from that trail. To prevent decomposition of the sample, the collected ants were kept in a glass vial in a -80°C freezer until extraction.

The entire ant bodies were then ground for 3 minutes in a pestle and mortar in 40ml diethyl ether. All the solid and liquid matter was then placed into a glass vial and sonicated with a probe sonicator for 2 minutes. This mixture was agitated with a vortex mixer for 1 minute and placed in a centrifuge for 3 minutes at 900 rcf. The supernatant was then transferred to a new vial and stored at -80°C.

The remaining bodies remnants were extracted in the same way a further two times, to ensure maximal extraction of pheromone. A bioassay (described below) was used to test whether the extractions could produce a trail following response in *L. flavus* foragers (nurses do not follow trail pheromone [see Chapter 2]). It was found that only the original extraction and the first repeated extraction produced trail following activity; test ants crossed of 3.5 and 2.5 median radii respectively, so these two extractions were combined for purification. The median radii crossed by test ants for a second repeated extraction was 0 radii, so this extraction was not used for further analysis.

In order to exclude attractive pheromones present in the heads of the ants from the analyses, the extraction process was repeated a second time with another sample of

ant workers, this second extraction contained approximately 4600 individuals with their heads removed prior to solvent extraction.

4.3.4 HPLC separation of whole ant extracts and identification of attractive fractions

Normal phase HPLC was used to separate the whole ant extracts into 80 fractions. A high performance liquid chromatograph (HPLC) consisting of a Waters 600 controller and a Waters 717 plus autosampler was used with a Waters Spherisorb column (3mm silica). Two solvents were used as the mobile phase; A – Hexane and B – 90% Hexane & 10% Isopropanol. A gradient flow method was used with the following profile:

Time	Flow (ml/min)	%A	%B	Curve
-	2	100	0	-
5	2	100	0	6
35	2	90	10	6
55	2	50	50	6
61	2	0	100	6
79	2	0	100	6
79.1	2	100	0	11

At 79.1 minutes the column was equilibrated in 100% solvent A for 20 minutes at a flow rate of 2ml/min. A fraction collector was programmed to collect 1 fraction a minute for 80 minutes, resulting in 80 separate 2ml fractions.

To determine which fractions contained attractive pheromones the circle assay described below was used. Initially 10µl aliquots from a group of every ten fractions (fractions 1-10, 11-20, 21-30 etc.) were combined and tested in the bioassay. When activity was detected in a group, 10µl of each fraction comprising that group was diluted to 100µl with hexane and then tested individually.

4.3.5 Bioassay to test for attractiveness of test substances to ants

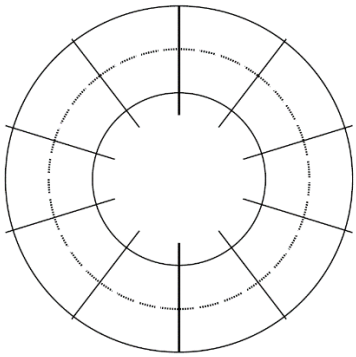

Fig.1 – The circular bioassay used to test potency of extractions at actual size. The radii intersect 1cm arcs on the centre (striped) concentric ring.

Extracts and test compounds were tested for attractiveness using the trail following bioassay developed in Chapter 3 of this thesis. The circular assay shown in fig.1 was printed onto standard printer paper at a size where the arcs of the dashed circle measure 1cm between the marked radii. Initially an ant was trapped in the middle of the centre circle using a small plastic enclosure coated with fluon to prevent escape. Trails of test substance were then laid on the dashed ring (second concentric circle) using a Hamilton syringe. Care was taken to ensure that 1µl of test substance was laid in-between each of the ten radii marked around the circle so the test trail was uniform and the total volume of test extract laid was 10µl. The ant was left for 40 seconds to habituate to the assay environment before release, the hexane carrier solvent evaporated after approximately 15 seconds. As the ant exits the inner circle it inevitably contacts the test trail and the number of radii crossed completely by the ant before it leaves either of the two bounding circles was counted. This was repeated on at least ten ants from a random selections of eight lab colonies

(lists of random numbers were generated using the RANDBETWEEN() function in Microsoft Excel), the median score of radii crossed then gave an estimate of attractiveness of the test substance. Results for test extracts were tested against results for a hexane control using Wilcoxon's signed rank test.

4.3.6 Identification of structures of potential recruitment in attractive fractions

Active fractions plus neighbouring fractions were analysed using a Thermo Trace GC Ultra gas chromatograph linked to a Thermo ITQ 1100 mass spectrometer. Helium was used as a carrier gas at a flow rate of 1.3ml/min. The column was an agilent DB-5ms UI, 30m x 0.25mm x 0.25µm film. The entire active fraction was blown down to 3µl, and 2µl were then injected into the GCMS using splitless injection. The GC oven was set to the following temperature programme; hold at 60°C for 4 minutes, ramp to 300°C at 10°C/min, hold at 300°C for 10 minutes. The mass spectrometer was used in EI mode (70eV), the scanning range was between m/z 40 and 650. Chromatograms were analysed using Thermo Xcalibur software (Thermo Xcalibur 2.3 build 26) and data were acquired in TIC mode, recording masses between 40 and 650 m/z. Compounds were tentatively identified by comparison with the NIST/Wiley database (NIST MS Search 2.0 g). Compounds were noted as potentially active if they were present in the fractions which produced a response in the circle bioassay and absent in the neighbouring, non-active fractions. To confirm the identity of potentially active compounds, synthetic standards were run on the GCMS to confirm retention time and mass spectra of the target compounds. If the identification was correct, the synthetic standards were then tested using the circle assay to ascertain if they were attractive to the ants.

Two compounds were identified from active post-HPLC fractions which also elicited a trail following response in test ants in the circle assay. 2,6-dimethyl-5-heptenol (DMH) was identified from the whole body extraction, and mellein was identified from the headless extraction.

4.3.7 Reduction of 2,6-dimethyl-5-heptenal

DMH was not available to purchase, so a synthetic standard was prepared by performing a reduction reaction with the corresponding aldehyde, 2,6-dimethyl-5-heptenal (97% purity). This was performed by dissolving the aldehyde in 1ml of a 1M NaOH aqueous solution at a concentration of 1mg/ml and placing the vial on ice. A sodium borohydride ($NaBH_4$) solution was also prepared by dissolving 10mg in 100µl ethanol. The $NaBH_4$ solution was added dropwise to the aldehyde solution. The vial was then taken off ice and left to react at room temperature for 20 minutes. 10 drops of 2M hydrochloric acid (HCl) were then added to the reaction vial to decompose the $NaBH_4$. A liquid-liquid extraction was performed by adding 1ml hexane to the reaction vial and mixing on a vortex mixer for 1 minute. The solution was left to partition for 1 minute, the top phase was then carefully transferred to a new vial using a glass pipette. The yield was then calculated by determining the concentration of aldehyde remaining after the reduction reaction took place using GCMS with citronellal as an internal standard. To do this, a standard solution was prepared containing 100ng/µl of 2,6-dimethyl-5-heptenal with 100ng/µl citronellal, 1µl of this was injected onto the GCMS instrument using the same temperature profile as described above and the relative abundance of 2,6-dimethyl-5-heptenal to citronellal was calculated. An aliquot of the post-reduction solution was diluted tenfold, and 100ng citronellal was added to

a 1µl aliquot of this dilute solution as an internal standard. This aliquot was then analysed by GCMS. Comparing the relative abundance of the aldehyde to citronellal to that of the standard gave a yield of 8.9%, revealing that 91.1% of the original amount was converted to the alcohol. In addition, the TIC signal for the alcohol in the post reaction solution was similar in intensity to 100ng of the aldehyde standard confirming that most of the 2,6-dimethyl-5-heptenal was reduced to the corresponding alcohol.

4.3.8 Quantification of attractive pheromones in ant bodies

Once a compound was identified as being present in active post-HPLC fractions and biological activity was confirmed with a synthetic standard, it was quantified in the bodies of ants using a calibration curve. DMH was quantified with a calibration curve ranging between 0.5-6ng on the column using 0.5ng phenyl acetate as an internal standard. Mellein was quantified using benzophenone as an internal standard and a calibration curve of 10-150pg mellein and 0.5ng benzophenone on the column. In order to determine the location of these compounds in the ant, extractions were made of ant heads and isolated hindguts. The hindguts of *L. flavus* are known to contain an attractive, previously unidentified, pheromone (Jones, 2014) and the heads of ants almost always contain alarm pheromones, which often elicit attraction in workers (Hölldobler and Wilson, 1990). Three head extractions were made, each from a different colony and each containing the heads of five randomly selected foraging workers extracted in 10µl of diethyl ether. Three hindgut extractions were also made from the same three colonies, each containing the hindguts of 20 randomly selected foraging workers extracted in 10µl of diethyl ether. All extracts contained phenyl acetate and benzophenone as internal standards at a concentration of 0.5ng/µl. Each

repeat extract was taken from a different ant colony. The heads or hindguts were crushed in ether for one minute using the tip of a glass pipette. The extractions were then placed into a sonicator bath for five minutes. 2µl of supernatant from each extract was then injected onto the GCMS using the same operating conditions as described above. In order to improve the sensitivity of the analyses, mass spectrometry data were collected using selected ion monitoring (SIM) or MS/MS modes rather than collecting a total ion chromatogram. The ion collection methods are shown in Table 2.

The decision to use SIM or MS/MS was based on which method produced the most

Target Compound	Time (mins)	Method	Masses Monitored
Phenyl acetate	8 - 9	SIM	51, 66, 94
2,6-dimethyl-5-heptenol	9.6 - 11	SIM	67, 81, 109, 142
Mellein	14 - 16.5	MS/MS	178 (parent): 160, 134 (daughters)
Benzophenone	16.51 - 17	SIM	77, 105, 182

Table 2 - Selected ion monitoring (SIM) and MS/MS conditions for the detection of identified pheromones and internal standards.

sensitivity and selectivity. Mellein was detectable at just 10pg on column when both MS/MS and SIM were used, but MS/MS also eliminated background noise in the chromatograms. When a TIC was collected, mellein was only detectable when 500pg was injected on the column. 2,6-dimethyl-5-heptenol was detectable when 50pg were injected on the column when SIM was used as the data collection method; this compares to 1ng on the column when a TIC was collected and 500pg when MS/MS was used.

4.3.9 Behavioural characterisation of pheromones – Trail function

To test for trail following behaviour of each identified pheromone (2,6-dimethyl-5-heptenol and mellein), the circle assay described above was used. The compounds were tested in the following concentrations: 1fg/µl, 10fg/µl, 100fg/µl, 1pg/µl, 10pg/µl, 100pg/µl, 1ng/µl, 10ng/µl and 100ng/µl. Hexane was used as a negative control and a hindgut extraction containing 10 hindguts in 100µl hexane was used as a positive control.

4.3.10 Behavioural characterisation of pheromones – Alarm function

In order to test for alarm function of each identified pheromone (2,6-dimethyl-5-heptenol and mellein), the Mandible Opening Response (MOR) assay was used. This is an established assay used to test for an alarm response of ants to a test compound by detecting a mandible opening behaviour (Guerrieri and d'Ettorre, 2008). 20 ants, randomly chosen from 6 lab colonies, were harnessed in plastic pipette tips using masking tape ensuring that only the head of the ant protruded from the tip of the pipette. The identity of each ant was recorded for use in statistical analysis. The ants were left for 2 hours to habituate to the harnesses. 1µl of a solution of each test compound in hexane was applied to a 2mm x 2mm square of filter paper and left for 25 seconds to allow the solvent to evaporate. The square was presented to a test ant at a distance of 1mm from the antenna and care was taken not to touch the antenna of the test ant. The compounds were tested in the following concentrations: 1pg/µl, 10pg/µl, 100pg/µl, 1ng/µl, 10ng/µl and 100ng/µl. Hexane was used as a negative control and a whole body of a non-nestmate worker was held in forceps 1mm away from the test ant antennae as a positive control. This was used to expose test ants to

the natural mixture of alarm pheromones that they would encounter in the wild as ants display full alarm behaviour when grasped by forceps. The controls and treatments were presented to the same set of 20 test ants in a randomised order.

4.3.11 Statistical analysis

All circle assay data were analysed used Wilcoxon signed rank tests as the data were not normally distributed. Ants were tested repeatedly with different treatments for the MOR assay, so generalized linear mixed models (GLMM) were used to analyse the data. The model included individual ant worker nested within colony as a random factor to account for individual variation of ant response in the analysis. The maximal model contained test compound concentration, compound tested and test order as fixed factors. As the MOR assay produces a binary response variable, treatments were compared using a binomial distribution and a logit-link function (the canonical link function for binomial response models). All factors were used in the final model, as there were no non-significant variables to remove. All statistical analysis was performed in R v3.1.3 (R Core Team, 2015).

4.4 Results

4.4.1 Circle assays to test for attractiveness of fractions from HPLC of whole body and headless extractions

After the whole body and headless extractions were fractionated using HPLC, circle assays were used to determine which groups of fractions produced trail following activity, these results are shown in Figures 2A and 2C. Figures 2B and 2D show the trail following activity produced by individual fractions from the active groups in 2A and 2C.

After the whole body extract was separated by HPLC, the only group of fractions which produced a trail following response significantly higher than the hexane control was 11-20 (Figure 2A) ($W = 84$, $p = 0.009$). When each of these fractions were tested individually fractions 18 and 19 produced a trail following response that was significantly higher than the hexane control (Figure 2B) (fraction 18: $W = 23$, $p = 0.037$, fraction 19: $W = 14.5$, $p = 0.006$). The median number of radii crossed by test ants when fraction 18 was tested was 4.5. When fraction 19 was tested the median response was 2.5.

When the groups of fractions produced by HPLC separation of the headless extract were tested, again the group containing fractions 11-20 was the only group to elicit a response that was significantly greater than the hexane control (fig. 2C) ($W = 90.5$, $p = 0.01$). When tested individually, fractions 18, 19 and 20 all produced a significantly greater following response than the hexane control (fraction 18: $W = 21.5$, $p = 0.025$; fraction 19: $W = 3$, $p = 0.0004$; fraction 20: $W = 9.5$, $p = 0.002$). The median radii crossed by ants when fractions 18, 19 and 20 were tested was 1.5, 5.5 (excluding an

outlier at 64 radii crossed) and 4 (excluding an outlier at 113 radii crossed) respectively. The median response to a hexane control was never above 1 crossed radius.

In terms of retention time, the activity profiles for Figures 2B and 2D (the individual fractions for whole body and headless extractions respectively) are directly comparable, i.e. when standard reference compounds were run on the HPLC

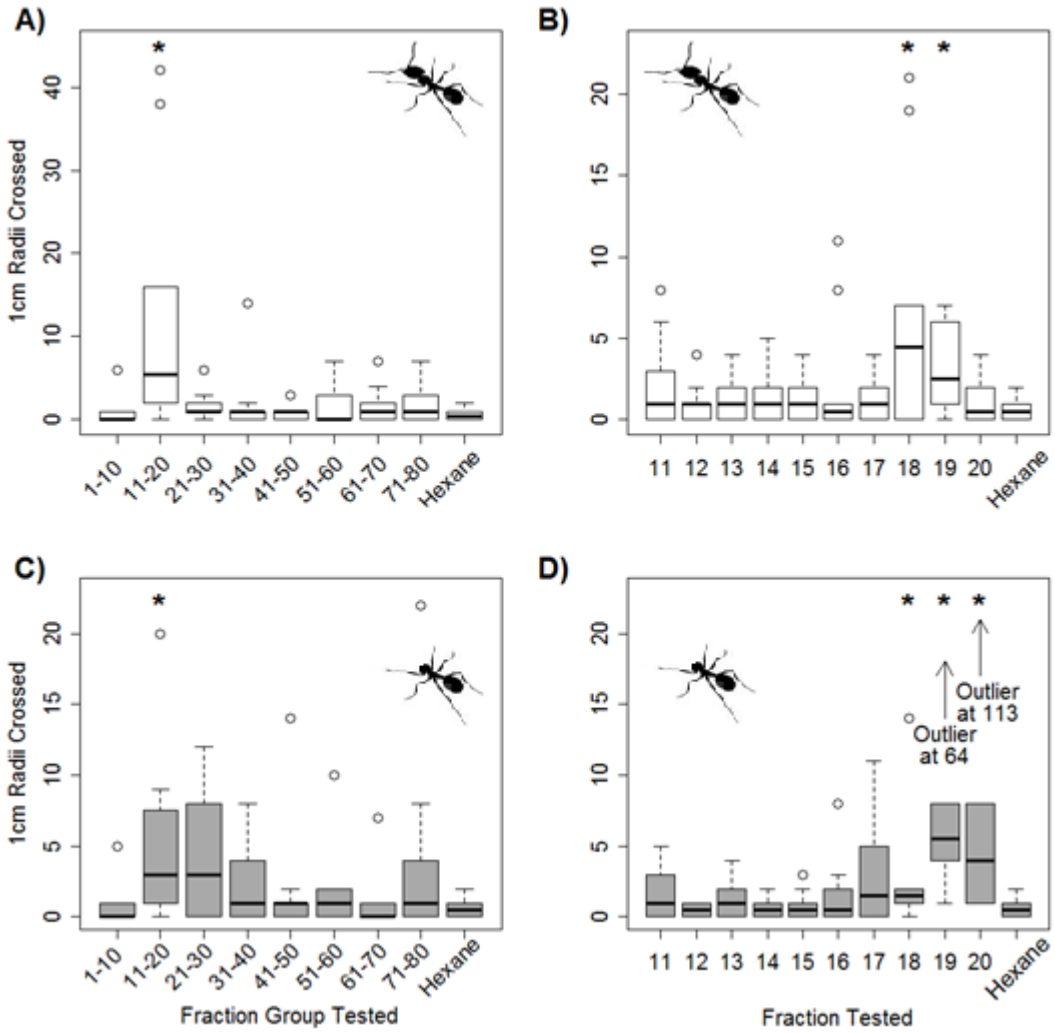

Fig. 2 – Behavioural assay results demonstrate HPLC fractions that elicited trail following behaviour. The median number of radii crossed by test ants using experimental trails of: **A)** Groups of HPLC fractions from the whole body extract, **B)** Single fractions from the active group of fractions from the whole body extract, **C)** Groups of HPLC fractions from the headless body extract and **D)** Single fractions from the active group of fractions from the whole body extract. Outliers were found at 64 and 113 radii crossed when fractions 19 and 20 were tested respectively. The * symbols show test extracts that elicited a response significantly higher than the hexane control ($p < 0.05$). Bars show median values, boxes show interquartile ranges, whiskers show extreme ranges and outliers are displayed as open circles.

instrument prior to separation of the extractions, the retention times exactly matched.

The most potent behavioural response to the whole body fractions was in fraction 18

(4.5 median radii crossed), whereas the most potent from the headless extraction separation was fraction 19 (5.5 median radii crossed). The median response to fraction 18 in the headless extraction was just 1.5 radii crossed. This indicated that an attractive compound in the head eluted from the HPLC at 18 minutes which was absent from the headless extraction.

4.4.2 Identification of an active compound from whole body extraction

After determining that fractions 18 and 19 from the whole body extraction produced a positive response in the circle assay (Figure 2B), GCMS analysis was used to investigate the chemical composition of these and neighbouring (inactive) fractions, so fractions 17, 18, 19 and 20 were injected onto the instrument for analysis. The GCMS chromatograms for these fractions were carefully examined, and only one peak was found that was present in fractions 18 and 19 and absent in fractions 17 and 20. It was present at retention time 9.93 in fractions 18 and 19 but was absent in fractions 17 and 20. This can be seen in Figure 3A, which shows the total ion chromatogram (TIC) for fraction 18, and Figures 3B-E, which show a section of the total ion chromatograms for fractions 17, 18, 19 and 20.

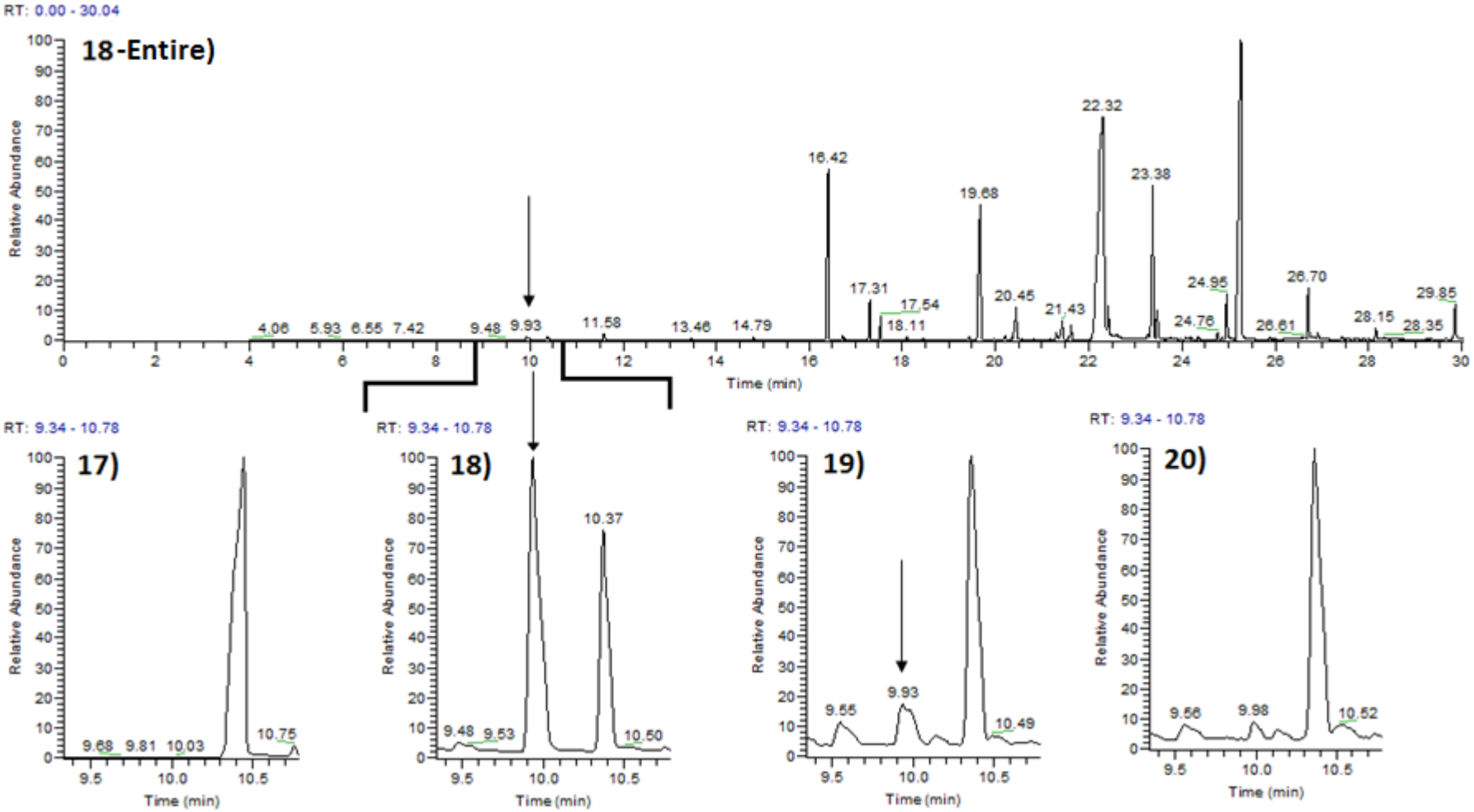

Fig. 3 – The presence of the peak at RT 9.93 corresponds with trail following activity elicited by HPLC fractions. The numbers refer to the fraction represented by the displayed chromatogram. 18-Entire) The entire TIC chromatogram for fraction 18 of the whole body extraction; the compound of interest is at retention time 9.93 minutes and is shown by an arrow. 18) Is a zoomed image of region from 9.34 – 10.78 minutes in chromatogram 18-Entire). 17), 19), and 20) show the corresponding region from the neighbouring fractions. The peak of interest is shown by an arrow and is present in 18) and 19) but absent in 17) and 20). Full chromatograms for fractions 17, 19 can 20 are shown in appendix IV.

Figure 4 shows the mass spectrum and corresponding chemical structure for the peak of interest which is highlighted in Figure 3. The initial loss of m/z 18 from the

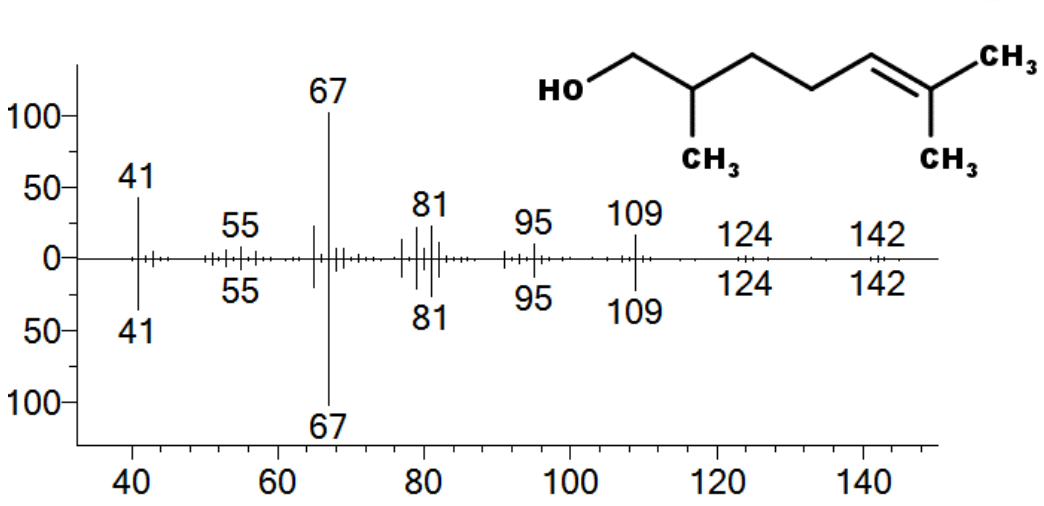

Fig. 4 – The mass spectrum of a synthetic standard matches that of the peak at RT9.93 in fractions 18 and 19. **Top plot –** The mass spectrum for the peak at retention time 9.93 in fraction 18 of the whole body extract which was identified as 2,6-dimethyl-5-heptenol. **Bottom plot** – The mass spectrum for a synthetic standard.

molecular ion of m/z 142 is likely to be the loss of H_2O, indicating the presence of a hydroxyl group. Further losses of m/z 14 and 15 indicate losses of CH_2 and CH_3 respectively. This spectrum was found to match that of 2,6-dimethyl-5-hepten-1-ol (DMH) (Law *et al*., 1965). The retention time and mass spectrum exactly match that of a synthetic standard (Figure 4). This standard was made by purchasing the corresponding aldehyde and performing a reduction reaction.

The results of trail following tests of the aldehyde, the alcohol and a hexane control can be seen in Figure 5. DMH elicits a significantly greater following response than a hexane control did when tested on ants in the circle assay ($W = 45$, $p = 0.014$); it produced a following response of 3.5 median radii crossed whereas a hexane control produced a median response of 0.5 radii. The equivalent aldehyde which the alcohol

was reduced from did not produce a trail following response (0.5 median radii crossed) (Figure 5).

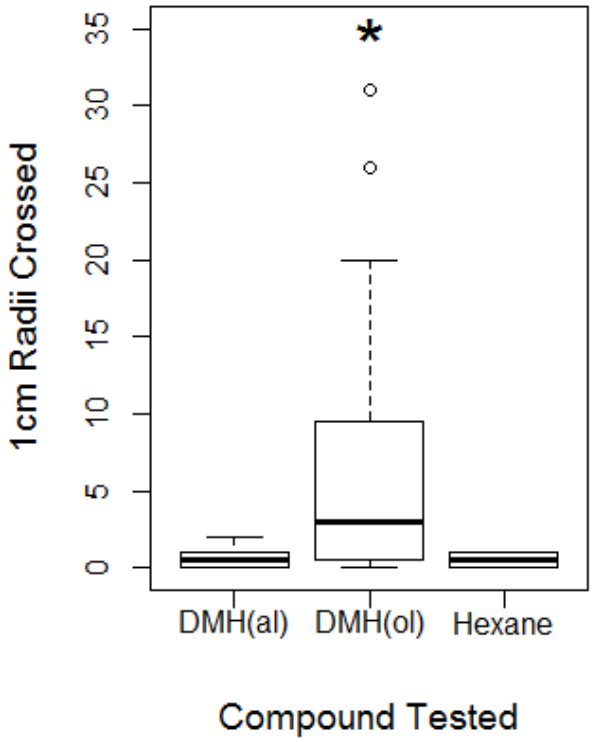

Fig. 5 – The median number of circle assay radii crossed by test ants when DMH and the equivalent aldehyde was used as the test substance at concentrations of 1ng/µl. The bars show median values, the boxes show the interquartile range and whiskers show the extreme range, outliers are displayed as open circles. The star designates that the response to DMH(ol) was significantly greater than to a hexane control ($p = 0.014$)

4.4.3 Identification of active compounds from headless extraction

After a potential recruitment pheromone was identified from the whole body extraction, GCMS analysis was also used to investigate the compounds present in the active fractions of the headless extraction. The fractions which produced trail following activity in the circle assay were fractions 19 and 20 (Figure 2D), so fractions 18, 19, 20 and 21 were analysed using GCMS. The chromatogram for fraction 19 (the most active) is shown in Figure 6. It shows a complex mixture of peaks, one of which was found in trace amounts in fraction 19 and absent in all other fractions. There were no other

peaks which could be potential recruitment pheromones, as they were all either present in the non-active fractions, or absent in fraction 19 (the most active). For this reason the trace peak was further investigated.

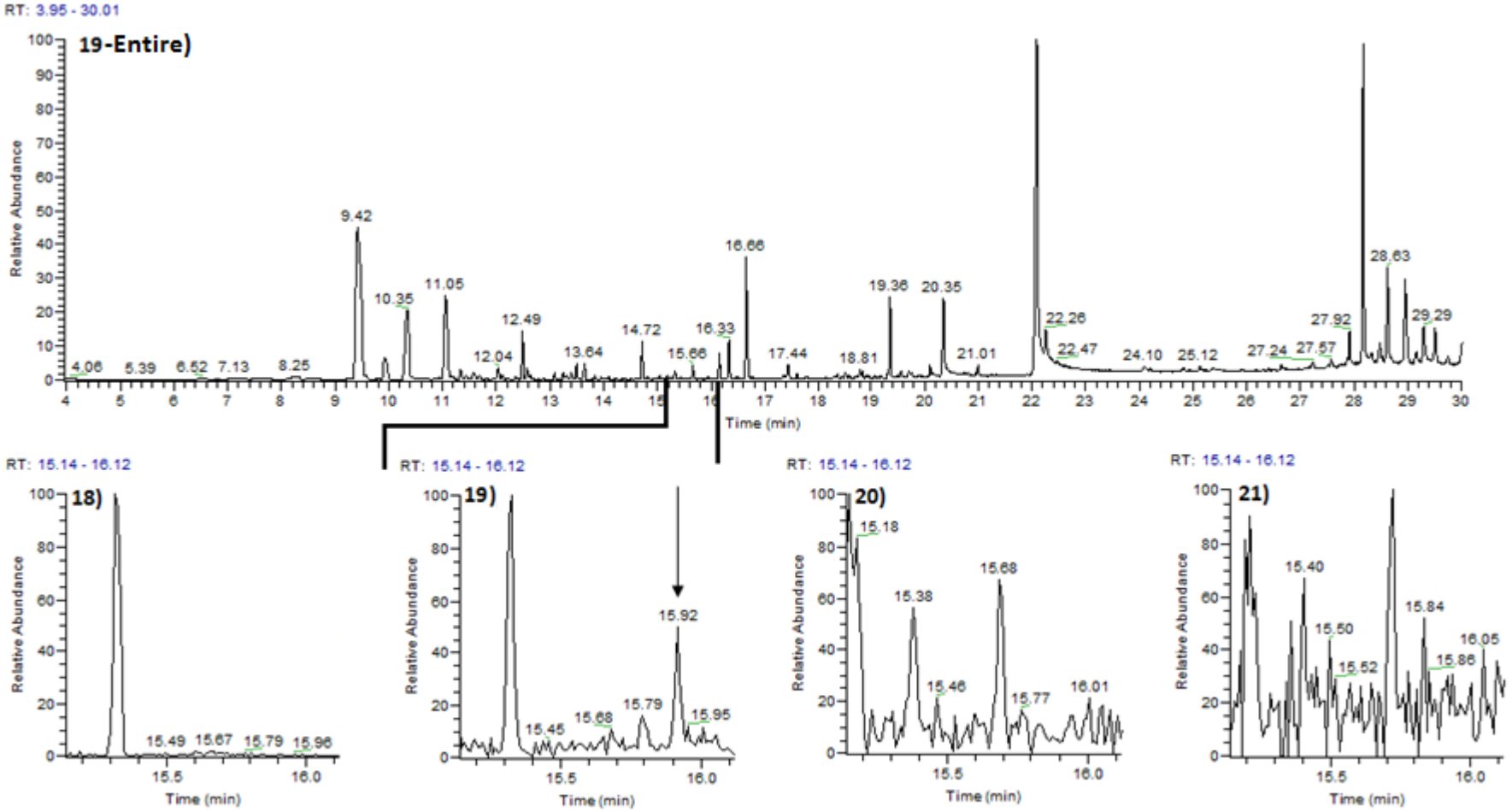

Fig. 6 – The presence of the peak at RT 15.92 corresponds with trail following activity elicited by HPLC fractions. The numbers refer to the fraction represented by the displayed chromatogram. 19-Entire) Shows the entire TIC chromatogram for fraction 19 of the headless extraction, the compound of interest is at retention time 15.92 minutes but is not visible on the TIC. 19) Shows a zoomed in, extracted ion chromatogram for ion m/z 178 over the region 15.14 – 16.12 minutes of the chromatogram in 19-Entire). 18), 20), and 21) show the corresponding regions from neighbouring fractions. The peak of interest is only visible in 19) and is shown with an arrow. Full chromatograms for fractions 18, 20 and 21 are shown in appendix IV.

The mass spectrum for this compound is shown in Figure 7, and exactly matches that of mellein, a compound which has been identified as the trail pheromone of the

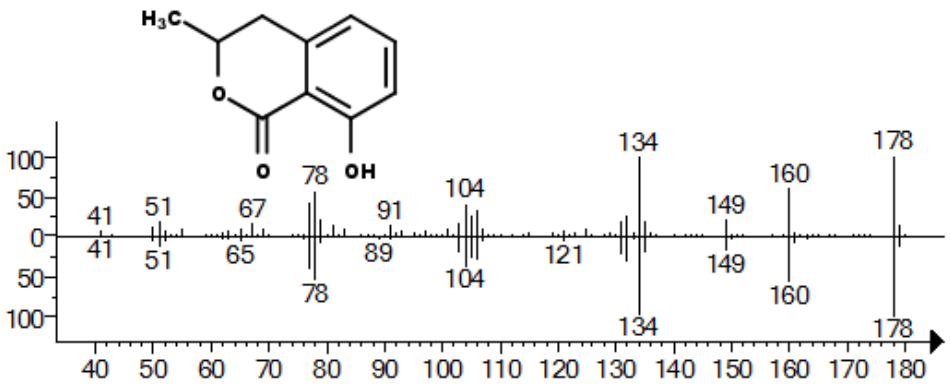

Fig. 7 – The mass spectrum of a synthetic mellein standard matches that of the peak at RT15.92 in fraction 19. **Top plot –** The mass spectrum for the peak at retention time 15.92 in fraction 19 of the whole body extract. Identified as mellein. **Bottom plot** – The mass spectrum for a synthetic standard.

congeneric shiny black ant, *Lasius fuliginosus* (Kern *et al*., 1997). The molecular ion of mellein is m/z 178, the initial loss of m/z 18 results in a peak at m/z 160. This indicates a loss of H_2O which represents the hydroxyl group. The next peak at m/z 149 is caused by a loss of m/z 29 which is likely to be a loss of CHO, corresponding to the ketone group on the molecule. A further peak on the mass spectrum is at m/z 134. This is a loss of m/z 44, which likely represents a loss of C_2H_4O; this corresponds to a loss of the methyl group from the molecule, along with the carbon it is bonded to and the neighbouring oxygen. This identification was confirmed by running a mellein synthetic standard (≥98% purity) which revealed an identical retention time and mass spectrum.

When tested in the circle assay, ants followed mellein significantly further than they did a hexane control (fig. 8) ($W = 76$, $p = 0.044$). The median response of ants to hexane was 0.5 radii crossed, whereas the median response to mellein was 2.5 radii crossed.

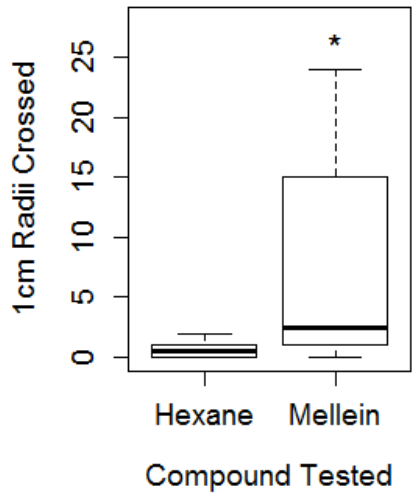

Fig. 8 – Ants followed mellein significantly further than they did a hexane control. Mellein was tested at a concentration of 1ng/µl. Bars show the median response, boxes show the interquartile range and whiskers show the extreme range. The star indicates the response to mellein was significantly greater than the response to a hexane control ($p = 0.044$).

4.4.4 Comparison of behavioural response to pure standards of identified pheromones using circle assay

The circle assay was used to test whether either of the two identified recruitment pheromones were likely to be trail pheromones. DMH and mellein were tested at a range of concentrations alongside a negative control of pure hexane and a positive control of a hindgut extraction (the hindgut is known to produce a trail following response in *L. flavus* and is the location of trail pheromone in other formicine ants). All test solutions were laid at a rate of 1µl per 1cm arc of the assay circle. The results of this assay are shown in Figure 9. The lowest concentration of mellein for which test ants showed a significant trail following response to was 0.01pg/µl, and the median

number of arcs crossed was 2.5 radii crossed. The response then increased to the maximal median response of 3.5 radii crossed when the test concentration was 0.1pg/µl. The response at increasing test concentrations did not show a clear dose-response relationship, as the median arcs crossed stayed approximately constant with increasing concentration. At the highest test concentrations of 10000 and 100000pg/µl the median response decreased to 1 and 2 radii crossed respectively.

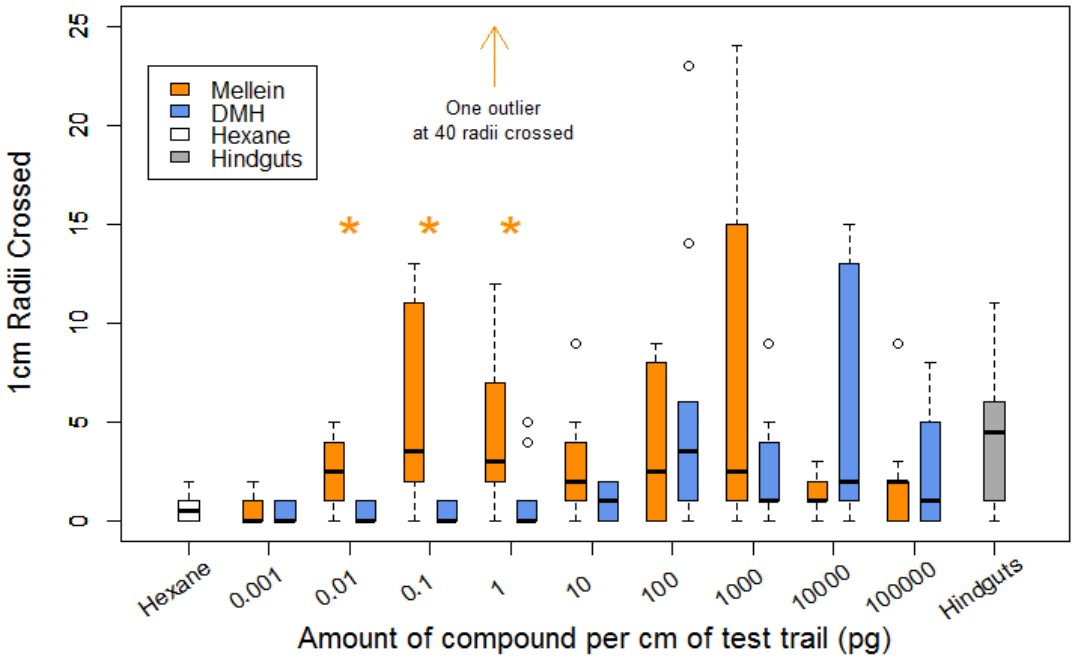

Fig. 9 – Ants followed mellein further than they did DMH and behaviour was elicited at far lower concentrations of mellein. The data show medians, interquartile ranges and extreme ranges, outliers are displayed as circles. The orange stars indicate that the response to mellein was significantly higher than the response to DMH ($p < 0.05$).

The response profile of DMH was very different to that of mellein. The first response was not detected until the test concentration reached 10pg/µl just 1 median arc crossed. The maximal response occurred when the test concentration was 100pg/µl and was 3.5 median arcs crossed. Again, there is no clear dose-response relationship present, but the trail following behaviour seemed to attenuate at the highest test concentration of 100000pg/µl with a median response of 1 arc crossed.

Mellein consistently produced a longer trail following response than DMH at concentrations lower than 100pg/µl, and this difference was significant when the concentration of test compound was 0.01pg/µl ($p= 0.013$), 0.1pg/µl ($p= 0.005$) and 1pg/µl ($p = 0.038$). There were no significant differences between the responses to the two test compounds at any other concentration. Neither of the pure pheromones produced the same level of response as the hindgut extraction at any concentration, suggesting that there may be a synergistic effect between the pure pheromones and other compounds in the extraction. The median number of radii crossed by ants tested with the hindgut extraction was 4.5.

4.4.5 Comparison of behavioural response to pure standards of identified pheromones using the mandible opening response (MOR) assay

The mandible opening response assay was used to test whether either of the identified pheromones was likely to be an alarm pheromone, as ants open their mandibles when alarmed. DMH and mellein were tested at a number of different concentrations and 1µl of test solution was used. A negative control of pure hexane and a positive control of a live and alarmed ant were also tested. The response rate is expressed as a percentage of 20 test ants which showed a MOR. These results are shown in Figure 10.

When mellein was used as the test substance, a mandible opening response was first detected when the concentration of test compound was 10pg/µl and the response rate was 5% of test ants exhibiting a MOR. A dose response relationship followed this, with the response rate gradually increasing with increasing concentration of mellein. The maximum response in the assay reached was at the highest test concentration of 100000pg/µl with 40% of test ants displaying a response.

When DMH was tested in the MOR assay, a response was first detected when the test concentration was lowest at 1pg/µl, the response rate was 5%. Again a dose response relationship was present, and the response rate increased to 65% at the highest test concentration of 100000pg/µl.

The proportion of ants which exhibited the MOR was always significantly higher when presented with DMH than with mellein, no matter the test concentration ($p = 0.002$, $\chi^2 = 10.1$). The highest overall response rate occurred when live, alarmed ants were presented to test ants, in this case 70% of ants displayed the MOR. Hexane produced a 0% response rate.

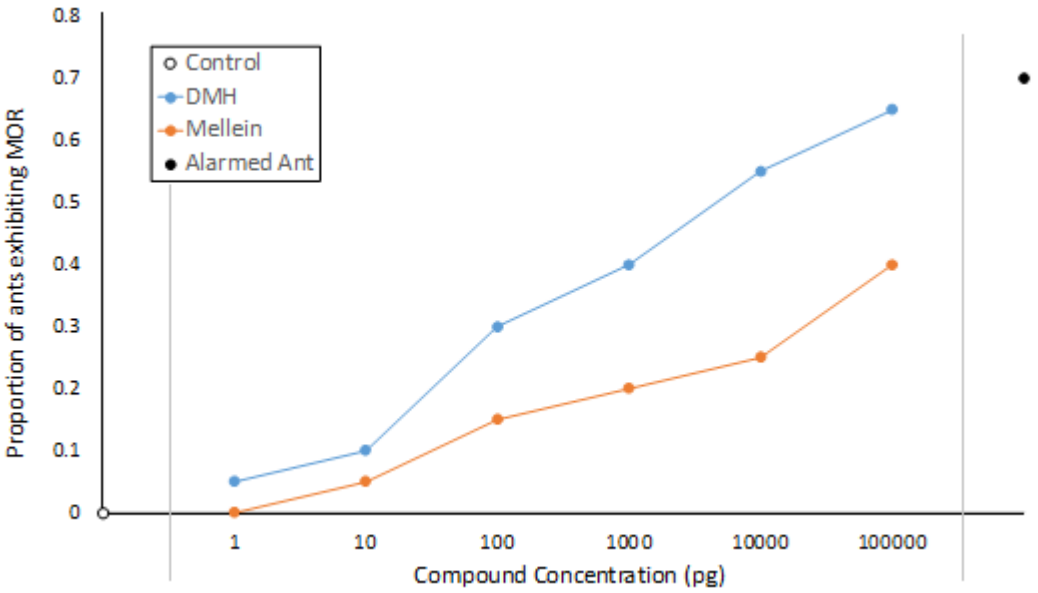

Fig. 10 – A higher proportion of ants performed the mandible opening response when exposed to DMH than they did to mellein. 20 were tested under exposure to DMH, mellein, hexane and ant bodies. DMH and mellein were tested at a range of concentrations.

4.4.6 Quantification of pheromones in extracts of ant heads and hindguts

In order to identify the source of the pheromone compounds, three repeat extractions were made of the most likely body parts to contain the identified compounds, ant heads and ant hindguts. Calibration curves were created to quantify each of the

pheromones in the two sets of extractions, benzophenone was used to quantify mellein and phenyl acetate was used for DMH (see Appendix V at the end of this thesis). Mellein was found only in hindgut extractions at a mean ± SD concentration of 5.56pg ± 1.4pg per hindgut and DMH was found only in head extractions at an average concentration of 1.12ng ± 0.58ng per head.

The hindgut extraction used in the circle assay when testing for trail following effect of the identified pheromones (the positive control in Figure 9) contained 10 hindguts in 100µl hexane. The quantification result indicates that the concentration of mellein in this hindgut extraction was 0.56pg/µl (5.56 pg/hindgut, 10 hindguts in 100µl hexane). In Figure 9, the median response by test ants to the hindgut extraction was 4.5 radii crossed. The approximately equivalent amount of mellein tested (1pg/µl) resulted in 3.5 median radii being crossed. These results were not significantly different ($p > 0.05$, $W = 50.5$), indicating that mellein accounts for all trail following behaviour present in the hindgut extraction.

Given the quantification for DMH above, in the MOR assay the maximum amount of DMH emitted by a non-nestmate ant (used as a positive control in Figure 10) cannot have been more than 1.12ng. The rate of MOR responses for non-nestmate ants was 70%, and when compared to the approximate equivalent amount of pure DMH (1ng) the response rate was 40%. Although a proportion test does not find a significant difference between these two values ($p > 0.05$, $\chi^2 = 2.53$), it is possible that DMH is not the sole component of the *L. flavus* alarm pheromone as the response rate to pure DMH does not reach the same level as when a live, alarmed ant is used as the stimulus. However, the effect of other stimuli affecting the test ant when a live ant was

presented (such as visual stimuli or pheromone being sprayed from the mandibles rather than volatilising from filter paper) cannot be ruled out.

4.5 Discussion

Using a novel combination of high resolution chromatography, robust bioassays and sensitive analytical chemistry two recruitment pheromones of the yellow meadow ant, *Lasius flavus,* have been identified despite, in the case of the trail pheromone, a minute amount (5pg per hindgut) of the target compound being present. Their behavioural functions have been compared and confirmed and their glandular sources identified.

The first identified compound, DMH, is a terpenoid compound with a terminal hydroxyl group. It has previously been identified as a volatile component produced by a variety of plants and fruits (Ding *et al*., 1994; Kurobayashi *et al*., 1991; Sato *et al*., 1990; Tomiyama *et al*., 2012), and it has been found in the heads of *Lasius claviger* (previously *Acanthomyops claviger*) males (Law *et al*., 1965) and workers (Regnier and Wilson, 1968). Although (Law *et al*., 1965) hypothesized that the pheromone may be a type of sex pheromone used to attract females, its presence in the heads of sterile workers refutes that hypothesis. Regnier and Wilson (1968) tested DMH in an attraction assay and found a strong response from workers to the compound, but did not discuss the significance of the finding. They also only tested workers using droplets of pure pheromone, and given the tiny amounts naturally produced by ants this was probably oversaturating olfactory receptors and affecting the ants' behaviour in a non-natural context.

Based on the findings presented here that DMH is present in the heads of *Lasius flavus* workers, and that the major gland present in the heads of formicine ants is the mandibular gland (Hölldobler and Wilson, 1990), it can be assumed that this compound is stored in the mandibular gland. The results of the circle assays show that *L. flavus* workers are attracted to DMH, and the MOR assay demonstrates a sensitive aggression response. For these reasons it is highly likely that DMH is an alarm pheromone of *L. flavus*. The MOR response when DMH was tested never quite reached the same rate as when an alarmed worker was used as the stimulus, even when it was tested in the most biologically relevant concentration of 1ng/µl (approximately equivalent to the amount present in a single head). This indicates that DMH is likely to be a component of a multicomponent alarm pheromone, as is the case for many other ant species (Hölldobler and Wilson, 1990). If this is the case, it is not known why other attractive pheromones were not detected when the circle assay was used to test the fractions after HPLC separation was performed on the whole body and headless extractions. Perhaps their effects were simply too subtle, or maybe the compounds were not at a biologically relevant concentration when the fractions were tested. Alternatively, other components of an alarm pheromone may have a synergistic effect with DMH and not be attractive in isolation, and were therefore not recognized by ants when presented to them individually. Another potential confounding factor may be cues presented by an ant as opposed to our experimentally presented pheromone, such as visual cues of alarmed ants, or the pheromone delivery method (sprayed from mandibles compared to volatilising from filter paper).

The second identified compound was mellein. It is a dihydroisocoumarin compound and was first discovered in the filamentous fungi *Aspergillus melleus* (Nishikawa,

1933). It has since been found in a range of animals and plants and appears to have antifungal, antibacterial and phytotoxic properties (Herzner *et al*., 2013; Hill, 1986). It has been previously identified as the trail pheromone of the congeneric ant species *L. fuliginosus* (Kern *et al*., 1997), as well as another formicine species, *Formica rufa* (Morgan, 2009). Mellein has also been found in the hindguts of three other formicine ants: *F. fusca, Camponotus silvicola and C. rufipes,* but did not elicit trail following behaviour (Morgan, 2009). Our study only found mellein in the hindguts of worker ants, where all formicine trail pheromones have been found thus far (Morgan, 2009), and it produced a very strong following response in the circle assay even at very low (sub-picogram) concentrations. The evidence strongly suggests that mellein is the trail pheromone of *L. flavus*. A hindgut extraction was tested in the trail following circle assay and from the quantification data it was calculated that the concentration of mellein in this extraction was 0.56pg/µl and the median radii crossed by test ants was 4.5. When compared with the closest test concentration of pure mellein (1pg/µl), the median response was 3.5 radii crossed. A Wilcoxon signed rank test found no significant difference between the two responses, and when the interquartile ranges shown in Figure 9 clearly overlap. This indicates that pure mellein explains all the trail following activity which is elicited by a hindgut extraction. This suggests that mellein is the sole trail pheromone of *Lasius flavus.* Furthermore, as mellein elicited trail following at a concentration of just 0.01pg/ml, it appears to be present at a concentration 50 times higher than is necessary to produce trail following behaviour.

Compared to the other two species in the *Lasius* genus which have had their trail pheromones identified, the amount detected in *L. flavus* was miniscule. *L. niger* hindguts contain 150-200pg of trail pheromone compound (see Chapter 3), and *L.*

fuliginosus hindguts contain 50-100pg (Kern *et al*., 1997). *L. flavus* hindguts only contained 5.56pg ± 1.4pg of mellein, tenfold less than the lowest estimate in *L. fuliginosus*. To our knowledge this is the smallest concentration of trail compound ever identified from an ant. *L. flavus* are also extremely sensitive to their trail pheromone, and are able to detect it at a trail concentration of 0.01pg/µl. Very few studies have investigated how sensitive ants are to their trail pheromones, but *L. flavus* appears to be the most sensitive to date. In general compounds are tested in nanogram quantities, which is biologically relevant if the species in question possesses similar quantities in their glands (Evershed *et al*., 1982; Jackson *et al*., 1990). Morgan and Keegan (2006) found that leaf cutting ants *Atta sexdens sexdens* could detect and follow their trail pheromone at a concentration of 1.5pg/cm of trail, but this is still 150x less sensitive than *L. flavus*. Other *Lasius* species may also be highly sensitive to extremely low concentrations of their trail pheromone substances, but this remains to be investigated. The low abundance of trail pheromone combined with the high sensitivity of workers may be an adaptation to the subterranean lifestyle of *L. flavus*. As the environment will reduce the rate of evaporation of any trail compounds produced by the ants, trails will persist for longer than they would on the surface of the ground. This means that *L. flavus* can reduce the amount of energy they expend on trail pheromone production by only laying very small amounts in trails.

Another important consideration of the results presented here is the similarity of behavioural responses when presented with an alarm and a trail pheromone. Both types of pheromone elicited some level of trail following and aggression (represented by the results of the MOR assay). It is only by comparing the behavioural responses to both pheromones in each assay that their functions were elucidated. This process was

not routinely performed in previous studies. Had either of the pheromones identified in this Chapter been tested using either assay in isolation (and without the guidance of congeneric pheromone identification providing information on likely chemical structures), misidentification of their primary function could easily have occurred. For this reason, the comparative examination of responses to putative pheromones in a range of concentrations in different bioassays should be always be considered.

The methodology used in this Chapter should allow for further advancement in the field of recruitment pheromone identification. Other pheromones that cause quantifiable behavioural responses could also be investigated by using different bioassays to test extraction fractions after HPLC has taken place, for example avoidance assays could be developed and used to detect 'stop' pheromones such as those shown to exist in Pharaoh's ants (Robinson and Jackson, 2005). Furthermore, this experimental method is not limited only to ants, but any other organism where sufficient quantities of extractions can be made and behavioural observations are achievable.

5 The alarm response of *Nasutitermes corniger* and colony level behavioural modification in the context of gallery repair

5.1 Abstract

After exploring the differences in responses to alarm pheromones and trail pheromones in ants in Chapter 4, I now go on to investigate alarm signals in termites. There is currently a lack of clarity as to exactly when alarm signals are released by termites and what their response to them is. The aim of the work presented in this chapter is to elucidate the true response of *Nasutitermes corniger* workers and soldiers to alarm, and to identify precisely when alarm signals are released. A further aim is to investigate whether any colony level effects exist when termites are exposed to alarm. These aims are investigated in the context of foraging gallery repair. When termite foraging galleries are broken, soldiers rush to defend the area while workers conduct the repair. Experimental breaks were made in foraging galleries and termites were exposed to two treatments; predation pressure simulated by a predatory ant (*Pachycondyla verenae*) and a control. Termite soldiers frequently responded to the presence of a predatory ant by firing a poisonous and sticky secretion from their frontal glands; 53 of 380 presentations (14%) of the ant stimulus elicited this reaction and it occurred at least once during every repair completed under the predation stimulus. Upon firing the secretion, the behaviour of termite workers was to immediately stop making repairs and flee into the safety of the gallery. This behaviour

was not observed when an ant was presented to the termites and soldier secretion was not fired, indicating that the point of alarm signal release was when the secretion was fired. The colony level effects detected when termites were placed under predation pressure was a slightly slower, more irregular repair. The particles used by termites in the repair were found to be larger when they were exposed to predation pressure, indicating that the repair may take overall fewer deposits. This did not represent a speed/accuracy trade off as is often found in nature, but rather a safety/accuracy trade off where the safety of workers was prioritised at the cost of a slower, more irregular repair.

5.2 Introduction

Eusocial insect colonies can appear to respond to stimuli as a single coordinated entity due to the emergent properties of many individuals following simple behaviours. Examples of this are swarms of honeybees collectively 'agreeing' on a new nest site (Beekman et al., 2006), or foragers from an ant colony switching food sources if a new source becomes more profitable (Grüter et al., 2012). In other words, in social groups individual behaviours often result in interesting group-level effects. It is therefore important to consider colony level behavioural changes alongside those of individuals when studying social insects.

Like all eusocial insects, termites utilise division of labour to share the workload faced by their colonies. The non-reproductive castes of termites are often morphologically and behaviourally specialised with regard to the tasks they need to perform (Bignell et al., 2011). The workers possess massively distended guts which contain symbiotic bacteria that digest the termite's major food source, wood. Termite soldiers are

perhaps the most specialised castes found throughout all the social insects as many cannot perform any task apart from nest defence. *Kalotermes* soldiers possess massively oversized jaws used to crush invaders, while *Termes* soldiers have oddly shaped mandibles which they can 'snap' together to incapacitate (and occasionally decapitate) arthropod enemies (Seid et al., 2008). Soldiers of the genus *Nasutitermes* are particularly unusual in that they possess no mandibles at all. Instead they possess a nozzle-like rostrum which is connected to a large frontal gland that produces a sticky secretion. Soldiers can fire this secretion out of the rostrum a distance many times their own body length, which entangles arthropod invaders. This secretion also acts as a topical poison and irritant, and may dissolve the cuticular waxes of arthropod invaders (LeBrun et al., 2014). This behaviour earned *Nasutitermes* soldiers the imaginative, but accurate description of 'perambulating artillery' by Maeterlinck and Sutro (1927). This peculiar morphology means that soldiers cannot assist with any other task required by the colony, indeed they cannot even feed themselves and must receive nourishment from the workers (Lima et al., 2014). *Nasutitermes* soldier glue consists of a mixture of a variety of monoterpenes and diterpenes (LeBrun et al., 2014). The exact constituents and quantities of the chemicals in the mixture varies by species (Prestwich, 1979a). The diterpenoid components give the secretion its adhesive properties, while the more volatile monoterpenes are thought to act as both solvent-carriers for the adhesive diterpenes and as alarm pheromones (Prestwich, 1979b), although this alarm function has not been extensively studied. The primary alarm pheromone in those species of *Nasutitermes* which have been studied is thought to be α-pinene (Moore, 1964). It is generally agreed that soldiers are attracted to this component of the frontal gland secretion, but there is conflicting evidence with regard to the response of workers. Stuart (1981) found that soldiers were attracted to the

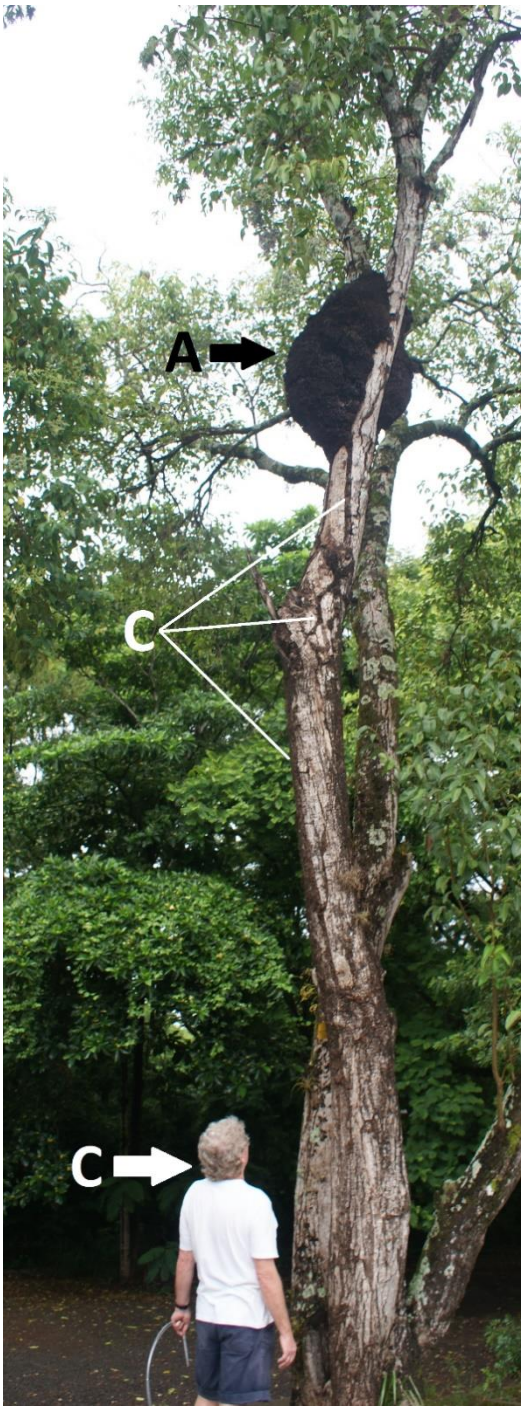

Fig. 1 – A) A *Nasutitermes corniger* nest; B) foraging galleries; C) A PhD supervisor provides a scale bar of 170cm.

cephalic secretion while workers retreated from it. This is in contrasts with the observations of Roisin et al. (1990), who found that both workers and soldiers were attracted to the secretion. Eisner et al (1976) found that workers did not respond at all to the secretion, while soldiers were attracted to it. One of the aims of this chapter is to clarify the response of *Nasutitermes* workers to alarm, and whether alarm behaviour is released at the point of soldiers firing their frontal gland secretion.

Termites of the genus *Nasutitermes* construct their nests in trees and build protective foraging galleries extending from the nest to foraging sites and down to the ground (see the image in Figure 1). The nest and gallery material is formed by combining faeces with pellets of dirt and small pieces of wood and stone (Bignell et al., 2011). The soft bodied *Nasutitermes* workers are almost entirely defenceless to predation when outside the galleries, so the protective galleries are essential to enable workers to forage for food and sustain the colony. If a break is made in a foraging gallery, soldiers rush to the opening in order to

prevent invaders from gaining access to the gallery and the nest itself. Workers will then attempt to repair the break by bringing material deposits to the site and using their faeces as an adhesive (McMahan, 1977). Some work has been done to describe the polyethism of different castes with regard to gallery and nest repair. McMahan (1977) found that young instar workers of *N. exitosus* did not participate in the repairs to nest mounds; most of the repair work was completed by workers of the oldest instar. Jones (1980) also found polyethism during gallery construction by *N. costalis*, but in this case, 1st instar small workers and 3rd instar large workers contributed the most to gallery repair. However, whether the repair process responds to environmental stimuli remains unstudied.

Speed/accuracy trade-offs are often present when individuals are placed under environmental pressure, as in nest selection site by *Leptothorax albipennis* ants. When faced with harsh environmental pressures, the ants lowered their quorum threshold to accept a new nest site (Franks et al., 2003). One aim of the study described in this chapter is to investigate whether termites are capable of modifying the gallery repair process in the face of environmental pressure, and whether they respond with a speed/accuracy trade-off by making faster repairs that are of poorer quality. The major predators of termites are ants (Bignell et al., 2011). Therefore environmental predation pressure was simulated using an ant species known to predate on termites, *Pachycondyla verenae.* As mentioned at the beginning of this chapter, it is important to study the colony level effects of behaviour in social insects, but to date the colony level effects of alarm on termites have so far been completely neglected by the literature. A speed/accuracy trade-off would represent an important colony level modification in behaviour.

To induce the repair process experimental breaks were made in the gallery walls. To investigate whether gallery repair behaviour is modified in response to predation pressure, the termites were exposed to two treatments: predatory *P. verenae* ants and a control. The speed and regularity of repairs in both treatments was measured. As termites are alarmed by the presence of predatory invaders, the behavioural response of workers and soldiers when alarmed was also observed. The aim of these observations was to elucidate whether alarm behaviour is released simply by the presence of a predatory ant, or if an alarm response is only observed when a soldier fires its secretion, which may contain alarm pheromones.

5.3 Materials and Methods

5.3.1 Study site and species

The study took place on the University of São Paulo campus in Ribeirão Preto, São Paulo state, Brazil (Coordinates: -21.162979, -47.860647). The experiments were carried out on seven wild *Nasutitermes corniger* colonies, which all had foraging galleries accessible from ground level. *Pachycondyla verenae* ants were used to elicit an alarm response in the termites. *P. verenae* workers were collected from the USP campus and kept in plastic tubs with fluon coated walls to prevent escape. The ants were fed on honey water, and freeze killed *N. corniger* soldiers and workers. Water was provided *ad libitum*.

5.3.2 Initiating gallery repair and recording the rebuild of foraging galleries

To initiate a rebuild of the termite foraging galleries, a 2cm break was made in a vertical section of the gallery, and all carton material was scraped off the surface of the

Fig.2 – *N. corniger* termite soldiers defending an experimental break in their foraging gallery during the ant treatment. The 2cm experimental break was made between the two black lines which have been overlayed onto the image. The *P. verenae* ant stimulus was held with forceps approximately 1cm away from the termites. The ant was held in place for 10 seconds and the treatment was repeated once every 2 minutes.

tree. The breaks were made at distances between 2 and 4m from the nest, this varied depending on how high the termite nest was in the tree. After the breaks were made, the termites were then exposed to one of two treatments during the rebuild. One treatment (the 'ant' treatment) was to hold a *P. verenae* worker with forceps adjacent to the exposed gallery section at a distance of approximately 1cm for 10 seconds. This was repeated once every 2 minutes until the repair was complete (when there were no remaining gaps in the gallery walls). Figure 2 shows the ant treatment in progress. The other treatment (the 'control' treatment) was to hold a pair of clean, empty forceps adjacent to the exposed gallery section for 10 seconds, once every 2 minutes until the repair was complete. Each colony was exposed to both treatments once, with the break made in the same position during each treatment. The treatments were presented in a random order which was generated using the RANDBETWEEN() function in Microsoft Excel. The colonies were left undisturbed between treatments for a period of at least 17 hours. The entire repair process was recorded on a digital camera to allow subsequent analysis.

5.3.3 Measuring the alarm response of *N. corniger*

To identify the source of alarm signals in *N. corniger*, the reponse of workers and soldiers was measured immediately after three different stimuli which occurred during the ant treatment in the experiment described above. The first stimulus was when a shot of glue was fired at the *P. verenae* worker by a soldier, the second was when a *P. verenae* worker was presented to the termites but no glue shot was fired and the third was no stimulus at all. This third stimulus was used as a control to measure whether termites responded at all to the first two described stimuli. Three responses were measured, the number of workers visible 5 seconds after the stimulus, the number of soldiers visible 5 seconds after the stimulus, and the number of deposits made by workers for 10 seconds after the stimulus. These three responses were measured for 16 of each of the three stimuli, and measurements were taken from a random selection of video recordings (and therefore random colonies) taken during the ant treatment.

5.3.4 Measuring irregularity of gallery repair

To measure the irregularity of the completed repair a photograph was taken of the side profile of the repair. A section of measuring tape was included in the photo directly above the repair to use a scale. Using ImageJ software (version 1.50i), a scale

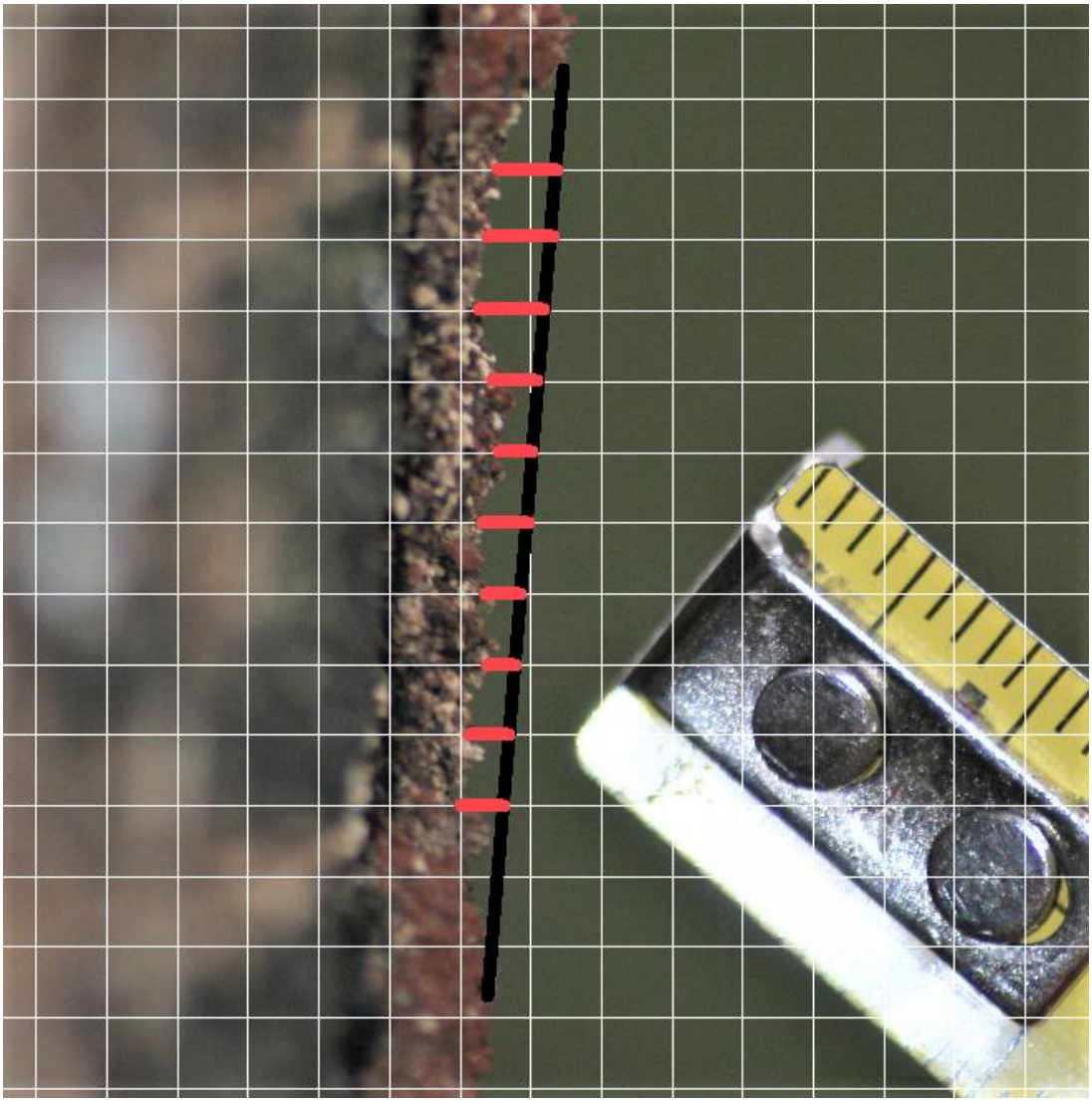

Fig. 3– The method used to measure irregularity of gallery repairs. The photo shows a side profile of a repaired gallery where termites were exposed to a predatory ant. The tree occupies the left of the image, and the termite gallery is visible on the surface of the tree, running down the centre of the image. The black line shows the profile of what would be a 'regular' repair, drawn by joining the two undistrubed areas of gallery before and after the break. The red lines show the deviation from that regular repair, measured at ten points along the repair's length. The points were selected by randomly overlaying a grid over the photograph, which is shown in white, where 10 horizontal lines intersected the repair, measurements were taken at those intersections. The mean of the deviation measurements (red lines) was the irregularity score for each repair, a perfectly regular repair would score 0. The tape measure was used as a scale. The length of each square represents 2mm.

for each image was set using the section of measuring tape. A line was then drawn connecting the gallery at each end of the repair, this line thus representing a completely 'regular' repair. The deviation of the repair from this line was then measured at ten points across the repair (one point every 0.2mm). A mean irregularity score was then calculated for each repair, and statistical tests were performed on these mean values. Figure 3 demonstrates how the measurements were made.

To try and identify a potential cause of irregular repairs, the size of particles used by termites in the repair was measured. This was done by randomly selecting sections of video footage from each colony's gallery repair video. The first particle deposited by a worker during each random section of video was measured, and this was repeated 70 times for each treatment. This resulted in 140 particles measured in total. The particles were measured by taking a snapshot from a video just after a particle was deposited. This snapshot was then analysed using ImageJ software. The 2cm break distance (marked on the tree before the break was made) was used as a scale, and the 'freehand' selection tool was used to trace the outline of the particle. This allowed the calculation of the area of each particle in mm^2.

5.3.5 Time taken to complete repair

The time taken to complete each repair was measured from the first deposit laid by a worker until the last gap in the gallery was filled. This was recorded in minutes.

5.3.6 Likelihood of soldiers firing their secretion

All instances of glue firing were recorded throughout the repair process for both treatments. The likelihood of a glue shot being fired was calculated by dividing the

number of stimuli triggering one or more glue shots by the total number of stimuli presented during each repair.

5.3.7 Statistical Analysis

All statistical analyses were performed in R version 3.1.0 (R Core Team, 2014).

The differences in response to the three different alarm stimuli (ant with a responding glue shot, ant with no glue shot, and no stimulus) were analysed using Wilcoxon signed rank tests as the data were not normally distributed. To correct for multiple comparisons, p-values were adjusted using the Bonferroni correction.

The repair irregularity was expressed as the mean deviation of the repair from a 'regular' repair as measured at ten points, this gave each completed repair a mean irregularity score. The distribution of these means was non-normal, therefore the difference between repairs made during the ant treatment and control treatments was analysed using Wilcoxon signed rank tests. All colonies were exposed to both treatments so a paired test was used in the analysis.

The distribution of particle sizes was also non-normal, so a paired Wilcoxon signed rank test was also used to test for a difference between particles used in the repairs during the ant and control treatments.

Finally, the time taken to complete repairs was not distributed normally so was also analysed using a paired Wilcoxon signed rank test to analyse the difference between the ant and control treatments.

5.4 Results

5.4.1 General observations of the repair process

When experimental breaks were made in the foraging galleries, 5-10 soldiers immediately rushed to the area and emerged from the now open gallery on either side of the break. The soldiers then retreated back into the galleries, and a calm period ensued where no behaviour was observed other than soldiers standing guard at the entrances to the gallery. Eventually, one or two soldiers would tentatively advance out of the galleries onto the now exposed surface of the tree, sometimes quickly retreating back into the nest. After a variable number of these tentative explorations and retreats, a soldier would traverse the break in the gap. Once the first traverse was completed an increase in activity was observed. More soldiers and the occasional worker began to traverse the gap. After a few minutes, workers began to deposit faeces and particles of earth, wood and tiny stones along the edges of the exposed area, while soldiers stood guard evenly spaced along the perimeter of the exposed zone facing outwards. Continued deposits built up until walls were formed along the sides of the exposed area, connecting the two openings in the gallery on either side of the break. The walls continued to be built up from either side until they met along the centre of the break and the repair was complete.

5.4.2 Source of alarm signal and alarm response behaviour in *N. corniger*

When an ant was presented to the termites that elicited a glue shot from a soldier, the behavioural effects were striking. There was an immediate retreat of workers from the exposed tree surface back into the safety of the gallery, which resulted in a drastic decrease in the rate of repair deposits following the glue shot. The soldiers did not

retreat as the workers did, instead they remained to guard the gallery entrances. When an ant was present and no shot was fired, there was no obvious response from the termites.

The results of the termite response to a soldier firing glue at an ant, the presence of an ant (with no shot of glue) and no stimulus at all can be seen in Figure 4.

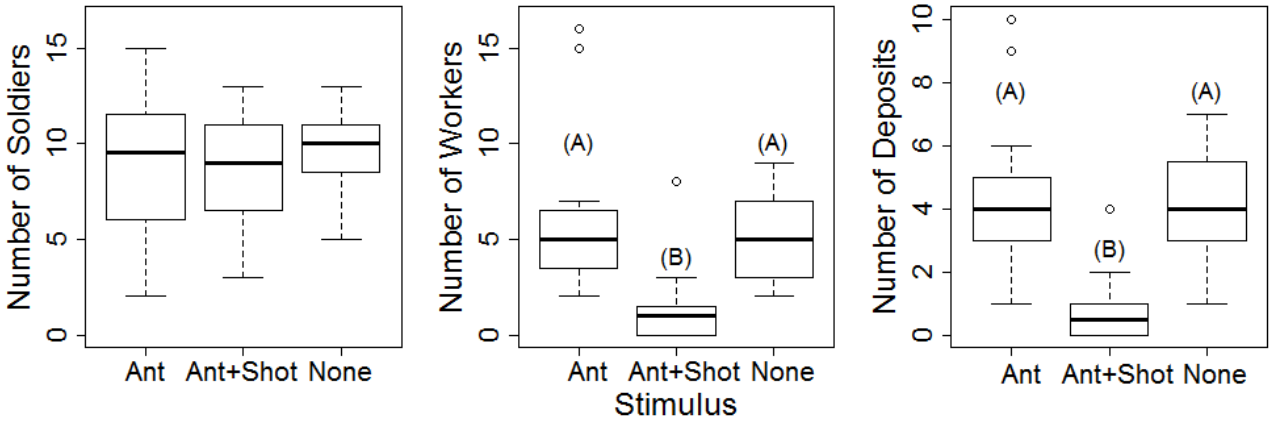

Fig. 4 – When a soldier fired a glue shot workers retreated into the gallery and stopped laying deposits, while soldiers remained outside. The graphs presented left to right represent: the number of visible nasutes present 5 seconds after each stimulus, the number of visible workers present 5 seconds after each stimulus, and the number of deposits made by workers in the 10 seconds following each stimulus. Letters indicate which responses were significantly different from other responses; results labelled (A) were significantly different to those labelled (B) (see text for level of significance). Bars represent the median response, boxes represent the interquartile ranges and whiskers show the extreme ranges of the data. Outliers are represented by open circles and were disregarded from subsequent analysis.

The median number of visible soldiers after an ant was presented was 9.5. When an ant was present and a glue shot fired the median number of visible soldiers was 9. When no stimulus was present at all the median number of visible soldiers was 10. There were no significant differences between any of these responses (ant:ant+shot, $p = 0.757$, $W = 119$; ant:none, $p = 0.58$, $W = 113$; none:ant+shot, $p = 0.282$, $W = 156.5$). These results indicate that an alarm was only raised when a soldier fired glue, and that the number of visible soldiers did not vary upon alarm.

The median number of visible workers after an ant was presented was 5, the same as when no stimulus was presented at all. Overall, these responses were not significantly different ($p = 0.75$, $W = 104$). However, five seconds after a soldier fired a shot, the median number of visible workers dropped to just 1, this was significantly lower than the response to an ant with no glue shot ($p < 0.001$, $W = 5$) and when there was no stimulus at all ($p < 0.001$, $W = 234$). These results show that the response of workers to alarm was to flee into the foraging gallery, rather than remaining outside as the soldiers did.

The median number of deposits made by workers during the 10 seconds after an ant was presented was 3.5. There was no significant difference to the median response of 4 deposits when no stimulus was presented ($p = 0.387$, $W = 91$). When an ant was presented and a soldier fired a shot of glue, the median number of deposits dropped to 0.5. This response was significantly different than when an ant was present and no shot fired ($p < 0.001$, $W = 15$) and when there was no stimulus ($p < 0.001$, $W = 238.5$). These results are in accordance with the number of visible workers after the stimuli and are unsurprising; when the workers flee into the gallery to hide from a threat, they can no longer make deposits to repair the break in the gallery walls.

5.4.3 Effects of predation pressure on gallery repair and likelihood of soldiers firing glue

Soldiers fired their glue secretion at least once during every repair performed under the ant treatment. No glue shots were observed at all during the control treatment. Overall, a predatory ant was presented to termites repairing their galleries 380 times throughout the entire experiment. 53 of those events were accompanied by a glue

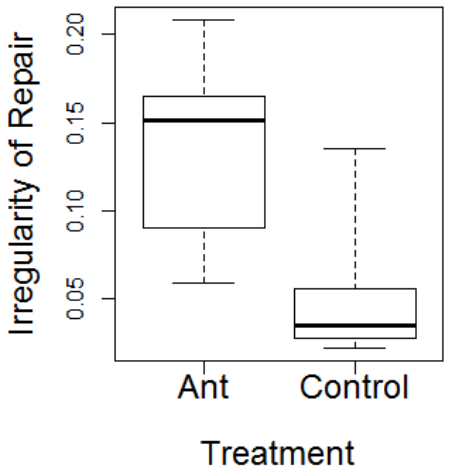

Fig. 5 – Repairs to galleries were significantly more irregular after the ant treatment ($p = 0.016$). The median, interquartile range and extreme ranges of average deviations from a 'regular repair' as measured at ten points along the repairs. Each of the 7 colonies was tested in the presence of an ant and a forceps control in a randomised, paired design.

shot from a soldier, representing an overall likelihood of a soldier firing a glue shot of 0.14, or approximately 1 time in every 7. When *N. corniger* termites repaired their foraging galleries in the presence of a predatory ant, the completed repairs were more irregular. The data are shown in Figure 5, and an image of the repairs made by the same colony of termites during each treatment is shown in Figure 6. The median irregularity score of the repair when ants were presented to the termites was 0.15, whereas repairs made during the control treatment had a median irregularity score of 0.03. This difference was statistically significant ($p = 0.016$, $V = 28$).

The particles deposited by termites were larger when the gallery was repaired in the presence of an ant, this is shown in Figure 7. The median particle size when an ant was present was 0.6mm^2, whereas the median particle size during the control repair was significantly smaller at just 0.3 mm^2 ($p < 0.001$, $W = 3814.5$).

The median time taken to complete the repair in the ant treatment was 100 minutes, whereas in the control treatment the median value was 84 minutes. The data are shown in Figure 8. The difference between the two treatments was not statistically significant ($p > 0.05$, $V = 24$).

Fig. 6 – Gallery repairs were more irregular when termites were exposed to the ant treatment. Both photographs show a side profile of a completed repair. The tree takes up the left side of each image, and the repaired gallery runs down the centre of each image. The tape measure was used as a scale. **Left** – a regular repair made by termites during the control treatment. **Right –** an irregular repair on the same tree made by termites exposed to the ant treatment. The lines visible on the tree show where the experimental cut was made in the foraging gallery.

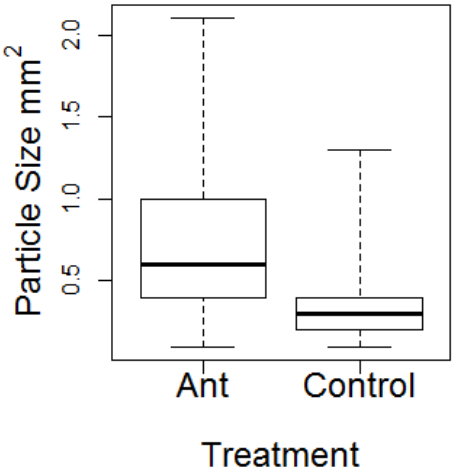

Fig. 7 – The particles used by termites in repairs were significantly larger when they were exposed to the ant treatment ($p < 0.001$). The bars show the median particle size, interquartile range is shown by boxes and the whiskers show the extreme ranges. 70 particles were randomly selected and measured for each

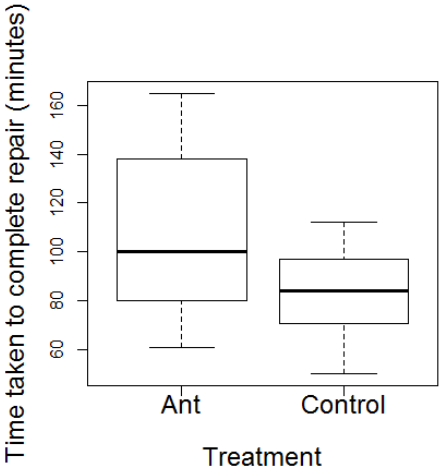

Fig. 8 – The time taken to complete the repair was not significantly different for either treatment ($p > 0.05$). The bars show the median completion time, the boxes show the interquartile range and the whiskers show the extreme range. Each of the 7 experimental colonies was tested once in each treatment.

5.5 Discussion

The result that there was no difference in the numbers of visible workers and soldiers, or the number of worker deposits made after an ant was present and when there was no stimulus indicates that the presence of ants alone was not sufficient to release alarm behaviour. However, when a soldier secretion was fired the number of visible workers quickly dropped, as did the number of deposits made by workers. This indicates two things with regard to the aims of this study. The first is that alarm signals are only transmitted when a soldier fires its secretion, and the second is that the response of *N. corniger* workers to this alarm is to retreat, thus confirming the observations of Stuart (1981). However, a caveat here is that I did not test the response of workers to soldier secretion in isolation of the soldiers themselves. It may be the case that the soldier secretion alone is attractive to workers and that soldiers may release another, as yet unidentified, pheromone when firing their secretion which causes workers to flee. Indeed it is common for pheromones to act as blends rather than in isolation (Hölldobler and Wilson, 1990). I also found no evidence that soldiers were attracted to the secretion as there was no difference in the number of soldiers present before and after secretion was fired; this contrasts with the work of Roisin et al (1990) and Eisner et al (1976) who found that soldiers were attracted to the secretion, though I note that their experimental methodologies were very different to those used here.

Soldiers were observed to fire their secretion at the predation stimuli used in this study, and evidence was discovered of alarm response behaviour thus indicating that the simulation of predation pressure was successful. There was also evidence of colony-level effects on the repair of foraging galleries; when under pressure of

predation, the completed repairs were far less regular in shape. This does not simply represent an aesthetic problem, as tunnel width has been shown to have an effect on the efficiency of termite movement. Tunnels of irregular width take longer for termites to navigate (Cho and Lee, 2014). The time taken to complete the tunnel repair was not found to significantly change between treatments, so it is unlikely that a speed/accuracy trade-off was taking place here. Though not statistically significant, it actually took termites slightly longer to repair tunnels when predation pressure was present, and so it seems that a safety/accuracy trade-off caused the observed results. As *Nasutitermes* soldiers do not represent any work resource to the colony when not defending the nest, they are less valuable than the workers with regard to feeding the colony; if a colony lost all its soldiers it could still sustain itself. When a predation threat presents itself, it is vital that the workers remove themselves from harm, and therefore in this experiment the building effort virtually stopped (see the number of deposits made after soldier secretion was fired in Figure 4). The response of termite workers to predation pressure (signalled by repeated alarm) was to use larger particles to repair the walls. Theoretically this means that the repair could be completed with less overall deposits, and allows them to spend more time out of harm's way with the emergent effect of a more irregular repair. This prioritises the safety of the workers while still allowing the gallery repair to take place. When the result of irregular gallery repairs became clear, and I formed the idea of a safety/accuracy trade-off, I attempted to record the overall number of deposits made during each repair to support this idea. Unfortunately, the sheer number and speed of deposits made when repairs were underway along with the unfavourable orientation of the video footage made this unfeasible. To truly confirm the idea of a safety/accuracy tradeoff, high speed footage would need to be recorded from multiple angles to record every deposit made by

workers, and the total number of deposits made would have to be compared when termites are under predation pressure. Presumably this safety/accuracy tradeoff would not have been observed if, by chance, ants were presented to repairing termites and no glue shots were fired, as only glue shots elicited alarm-response behaviour.

To summarise, if a *Nasutitermes* soldier detects the presence of a predatory ant it will fire its frontal gland secretion at the potential invader. Then one of two things happens, either monoterpenes in the secretion act as an alarm pheromone, causing workers to flee, or the soldiers simultaneously release another pheromone that acts as an alarm pheromone. The repeated alarm signals cause the workers to spend more time hiding and less time building, and they compensate for this somewhat by increasing the size of the particles they use to repair the break in the wall. This has the emergent effect of increasing the irregularity of the repair, which ultimately may reduce the speed that termites can move through the repaired section, but has the advantage of keeping workers safe while the nest is exposed. I note that the time of soldier frontal gland secretion release is concurrent with the release of an alarm signal from the solider, and that the behavioural response of workers to this signal is to retreat. I also discovered that repeated alarm signals result in a safety/accuracy-trade-off in the repair of the nest, which in turn leads to a sub-standard repair which may have consequences on termite movement efficiency. This safety/accuracy trade-off allows termite workers to retreat to safety when a threat presents itself, thereby securing the future potential of the colony to forage and sustain itself.

Nasutitermes termites are a major pest that can cause severe damage to structures and have adapted well to thrive in urban environments. They are found from Mexico down to Northern Argentina and in the city of Belem, Brazil, 50% of all insect-related

structural damage is caused by *Nasutitermes* species, with the single most important pest being *N. corniger* (Constantino, 2002). Insect repellents are becoming less effective as populations evolve resistance to many of the chemicals used (Deletre et al., 2016). Here, I show that the alarm signal in *N. corniger* has a negative effect on their ability to repair breaks in their foraging galleries. If the alarm pheromone involved can be identified, a worthy avenue of study would be to investigate whether this negative effect could be enhanced if the pheromone was applied in higher concentrations. If so, an effective repellent would be discovered which would be difficult for termites to become resistant to, as it is utilized in their own communication system. The technique of using insect pheromones as biological control agents was first recognised as a potential alternative to classic chemical control agents in 1967, and although results have been mixed, there are some success stories (Baker et al., 2016; Lin et al., 2016). Perhaps the use of termite alarm pheromones as deterrents could become one of them.

6 Discussion and Future Directions

6.1 The Chemistry of Formicine Ants

The work presented in Chapter 2 demonstrates that the conventional methods of gland dissection, liquid extraction and GCMS were not sufficiently sensitive to identify the trail pheromone of *Lasius flavus*. However, 27 other peaks were detected in extractions of poison glands and Dufour glands. A series of carboxylic acids was identified from the poison gland, but their function remains a mystery. The compounds detected in the Dufour gland were mainly alkanes and alkenes; this corresponds with those identified in the Dufour glands of other Formicine species (Chen et al., 2013; Haak et al., 1996; Hölldobler and Wilson, 1990; Lloyd et al., 1989). Of particular note note was the high concentration of the lactone micromolide and its homologues.

Other Formicine ants have had high abundance ketones and esters identified in their Dufour gland secretions (Attygalle et al., 1987; Bagnères et al., 1991; Haak et al., 1996), but microlomide is the first compound found in Formicine Dufour glands which has been proven to have proven antibacterial effects, albeit in other insects (Herzner et al., 2013). This suggests a potential new function for the Dufour gland in Formicine ants, that of an antibacterial reservoir. The antibacterial lactones found in the Dufour gland may act in concert with the antifungal formic acid (Tragust et al., 2013) from the poison gland to form a powerful antibiotic cocktail which the ants can use to disinfect their nests and brood. *L. flavus* may be unusual in the use of an antibacterial compound in its Dufour gland secretion, or it may turn out that all Formicine Dufour gland secretions possess some level of antibacterial action.

Another important finding was the cross contamination detected in extractions of compounds from neighbouring glands, despite careful measures to prevent it. Micromolide was the most abundant compound in all three of the gland extractions. Therefore if the glands were studied in isolation, it would be easy to conclude that the wrong gland was the source of this compound. It was only by comparative investigation that the Dufour gland was identified as the source of microlomide because Dufour gland extractions clearly contained the highest concentration of this compound. This has an important implication for future studies; when investigating the chemical contents of glands it is vital that the contents of neighbouring glands are also examined. This should also be kept in mind when interpreting previous studies that have examined glands in isolation.

6.2 Biochemical Polymorphism and Temporal Polyethism in *Lasius niger*

Chapter 3 went on to explore whether the chemicals present in the hindgut of *Lasius niger* vary depending on the temporal caste they belong to. Initially it was found that when foragers were tested on extractions made of nurse and forager hindguts, they followed forager hindgut extractions significantly further. This indicated that the concentration of trail pheromone did indeed differ between the two castes and was more abundant in foragers than nurses. Multivariate models then detected 14 compounds (including the trail pheromone) that were at a higher concentration in forager hindguts, and 4 compounds that were at a higher concentration in nurse hindguts. The compounds in the forager hindguts appear to be related to forager-type tasks such as nest defence and foraging, while the nurse compounds may be related to brood care. This is compelling evidence that the chemical toolkit of ant workers is

suited to the tasks performed by the caste they belong to, even when the ants are morphologically identical.

Given the result of the first chapter, that cross contamination does occur from other glands, some of these compounds probably originate in glands that neighbour the hindgut (such as the poison and Dufour glands). A clear follow-up to this study would be the comparative analysis of compounds in these neighbouring glands to confirm the glandular sources, and to potentially detect more compounds that characterise the chemical toolkit of nurse and forager castes.

Another finding of note is that the trail pheromone of *L. niger* was detectable using conventional dissection, liquid extraction and GCMS. This indicated that the failure to detect the trail pheromone of *L. flavus* in Chapter 2 was probably not due to some methodological error, but most likely because it is present in a lower concentration than that of *L. niger*.

It was also interesting to note that nurses did not follow hindgut extractions at all. This poses some new questions which have not yet been investigated. Do nurses possess the necessary receptors to detect trail pheromone or do these develop as the ant ages? If so, this may partly explain the regulation of temporal polyethism. Perhaps trail-following behaviour is not innate, but may be a behaviour that must be learnt in the presence of experienced ants as suggested by Cammaerts (2013).

6.3 The Identification of Recruitment Pheromones in Formicine Ants

The methods presented in Chapter 4 describe a successful attempt to overcome the issue of probable low concentration trail pheromone in *L. flavus,* which resulted in the

identification of two recruitment pheromones. To overcome the problem of low concentration, a mass-extraction technique was developed. As this extraction was made of whole bodies rather than just single glands, it contained many more compounds than a single-gland extraction. The compounds in this mass extraction were subjected to high resolution separation via HPLC, producing 80 fractions. A statistically rigorous trail-following bioassay (developed in Chapter 3) was then used to detect which of the fractions contained attractive chemicals. The finding in Chapter 3 that nurses did not follow trail pheromone extractions was vital here, so only foragers were tested to ensure the results were meaningful. The attractive fractions were then analysed via GCMS, which allowed further chromatographic separation and identification of target analytes.

The attractive compound was identified as 2,6-dimethyl-5-heptenol (DMH). When tested using an authentic standard, the trail following assay confirmed that workers followed trails of this compound. However, the structure did not resemble that of usual Formicine trail pheromones (dihydroisocoumarins or lactones), but its classification as an alcohol meant it resembled the alarm pheromones found in the mandibular glands of other ant species. Subsequent analysis of separate body parts detected a high concentration of DMH in the heads of the ants, indicating the source was the mandibular gland. This suggested that DMH was probably an alarm pheromone, because the mandibular glands of other species almost always contain these compounds (Hölldobler and Wilson, 1990).

This discovery led to another mass-extraction being performed without heads to exclude mandibular gland compounds, therefore making it more likely that the trail pheromone could be detected. This time, mellein was detected in the bioactive HPLC

fraction. This compounds is the trail pheromone of the congener *Lasius fuliginosus*. As with DMH, workers followed trails of this compounds but were much more sensitive to lower concentrations, thus indicating that this was the trail pheromone. To confirm DMH's identity as an alarm pheromone, the two compounds were tested on a relatively new bioassay developed by Guerrieri and d'Ettorre (2008) called the Mandible Opening Response (MOR) assay. Again the response of ants to both pheromones was similar, but ants were more sensitive to DMH than mellein at all test concentrations, thus indicating that DMH is indeed the alarm pheromone.

An important consideration here is that both pheromones elicited a positive reaction in each bioassay. If DMH had only been tested in a trail following assay, and without the consideration of previous Formicine pheromone structures, it would have been very easy to falsely conclude that it was a trail pheromone. Also, if mellein had been tested solely in the MOR, it may have been misidentified as an alarm pheromone. These results demonstrate that simply identifying an interesting chemical and testing it in one bioassay is not sufficient to identify pheromones and their natural functions. Many things need to be considered, including likely chemical structures based on those detected in related species, the location of the pheromone in the body, and comparative study involving bioassays that test for a variety of behaviours. Only then can one state the most likely function of the pheromone.

Perhaps the most important achievement in this thesis was the detection of the minute concentrations of trail pheromone in *Lasius flavus*. By using a combination of sensitive rigorous bioassays and analytical chemistry techniques, the lowest concentration ant pheromone (6 pg per gland) to date has been successfully detected and identified. Although it was disappointing that the compounds discovered were not

novel, this method paves the way for the identification of trail pheromones in other species which also utilise low concentrations. One example is the congener *Lasius neglectus*. This ant is an emerging pest species throughout Europe which outcompetes native ants and can have a disastrous effect on species diversity (Seifert, 2000). This ant occupies habitats alongside native species, so control with the use of broad spectrum pesticides is difficult as the non-pest species will also be affected. If the trail pheromone of *L.neglectus* can be identified and combined with an effective pesticide, a powerful, selective method of pest control may be achievable, which would leave native species relatively unharmed.

6.4 The Alarm Signal and Response of *Nasutitermes corniger*

Before pheromones are identified, the researcher should first observe a behaviour that they believe is released or modified by a pheromone. The work presented in Chapter 5 investigated alarm signalling and alarm response of workers and soldiers of the termite, *Nasutitermes corniger.* It was found that alarm is signalled at the point that soldiers fire secretion from their rostra, indicating that the frontal gland secretion itself contains alarm pheromone, or that soldiers simultaneously release an alarm pheromone from another gland. Another finding was that polyethism is present between workers and soldiers in their responses to alarm: soldiers did not noticeably respond to the alarm signal, while workers quickly fled and stopped repairing the experimentally broken foraging galleries.

Colony-level effects of alarm were also observed. The repairs made by termite colonies that were under predation pressure, and therefore releasing alarm signals, were far more irregular than those colonies that were undisturbed during the repair process.

This was driven by the use of larger soil, wood and stone particles that termites used to repair the wall. Overall this may decrease the number of deposits required to complete the repair, which may reduce the time workers expose themselves to danger, although this remains to be investigated. Irregular repairs represent a potential cost as termites cannot move through narrow tunnels as efficiently as they can wider ones.

As the repairs performed under predation pressure took longer (although not significantly so) than those undisturbed repairs, this behavioural modification does not represent a speed/accuracy trade-off. Instead it may represent a safety/accuracy trade-off whereby the termites prioritise the safety of workers over the most regular (and therefore efficient) completed repair. More work must be done to confirm this, such as counting how many deposits were actually laid over the course of a repair. This would require high speed video footage taken from multiple angles to ensure that every deposit is recorded.

It appears that termite alarm pheromones may be somewhat disruptive to the repair process of foraging galleries. A future avenue of study would be to identify exactly what the alarm compounds are, and then to investigate their effects on repair at higher than natural concentrations. If they completely prevent nest repair, a potential use may be as a natural repellent. It would be difficult for resistance to the repellent to evolve, as the termites use it in their own communication system. Termites represent a huge economic pest in many parts of the world, so novel methods of control would be a welcome development of this line of work.

6.5 The Evolution of Pheromones

A topic somewhat related to the work presented in this thesis that is worth investigating is the evolution of pheromones. Once a sufficient number of a particular type of pheromone has been identified, it may become possible to look for relationships between the environment a pheromone evolves in and its chemical structure. Take trail pheromones as an example. Ant species which use trail pheromones inhabit a vast range of very different environments. For example, different species thrive in tropical, arid and temperate climates. Presumably this environmental variation drives the evolution of the chemical structures used as trail pheromones. A very volatile trail pheromone wold not function well in a hot climate, as it may evaporate before the trail can be utilised to exploit resources. In a colder climate, a pheromone that was too involatile may persist for too long, fixing colonies into sub-optimal foraging patterns.

Though not direct, some related evidence for this was found by the work presented in Chapter 4. The concentration of trail pheromone used by *L. flavus* is the lowest detected in any ant so far at just 6pg per gland. This may be because *L. flavus* forages almost exclusively in underground tunnels. The flow of air through these tunnels will be much lower than above ground, and as a consequence the rate of trail pheromone evaporation will also be relatively low. Perhaps this means that *L. flavus* can use less trail pheromone than surface-foraging congeners, and therefore doesn't waste energy synthesizing more pheromone than is necessary.

Other factors may also influence the evolution of trail pheromones. For example a high density of closely related species may drive the evolution of a higher degree of

variations of the basic structure of a pheromone. It would be detrimental for one species to follow the trail of another, as ants tend to be highly territorial and following a trail to another species' nest may well result in death or injury. The review published by Morgan (2009) demonstrates that many species produce multiple variations of the trail pheromone compound, so the framework for selection from a variety of compounds has already been demonstrated. Another example of an evolutionary driver may be the primary food source of the species. Ants that forage on mainly sessile resources, such as leaf-cutting ants, could afford to have long-lasting involatile pheromones. Species whose food sources are ephemeral, such as those that scavenge for dead insects, would need to use more volatile pheromones so their resource exploitation remains flexible in a rapidly changing environment.

To investigate, this a database could be created containing all identified trail pheromones, along with the environmental conditions encountered by the species that use them. Other relevant information should be included, such as foraging systems and primary food sources. If not already known, the chemical properties of the trail pheromone compounds (such as volatility) could be estimated behaviourally or by chemical analysis. Statistical models could then be used to explore where important relationships lie between the trail pheromone compounds used and potential evolutionary driving factors. Once this step has been done, computer models could be used to explore the performance of different pheromone in a range of environments, including different temperatures, humidities and food sources.

The data may already be out there to explore this theme, and a positive thought is that the data resource will become ever stronger as more and more pheromones are

identified. My closing thoughts here likely echo those of countless PhD students before me and many more to come, ‘if only I had more time’.

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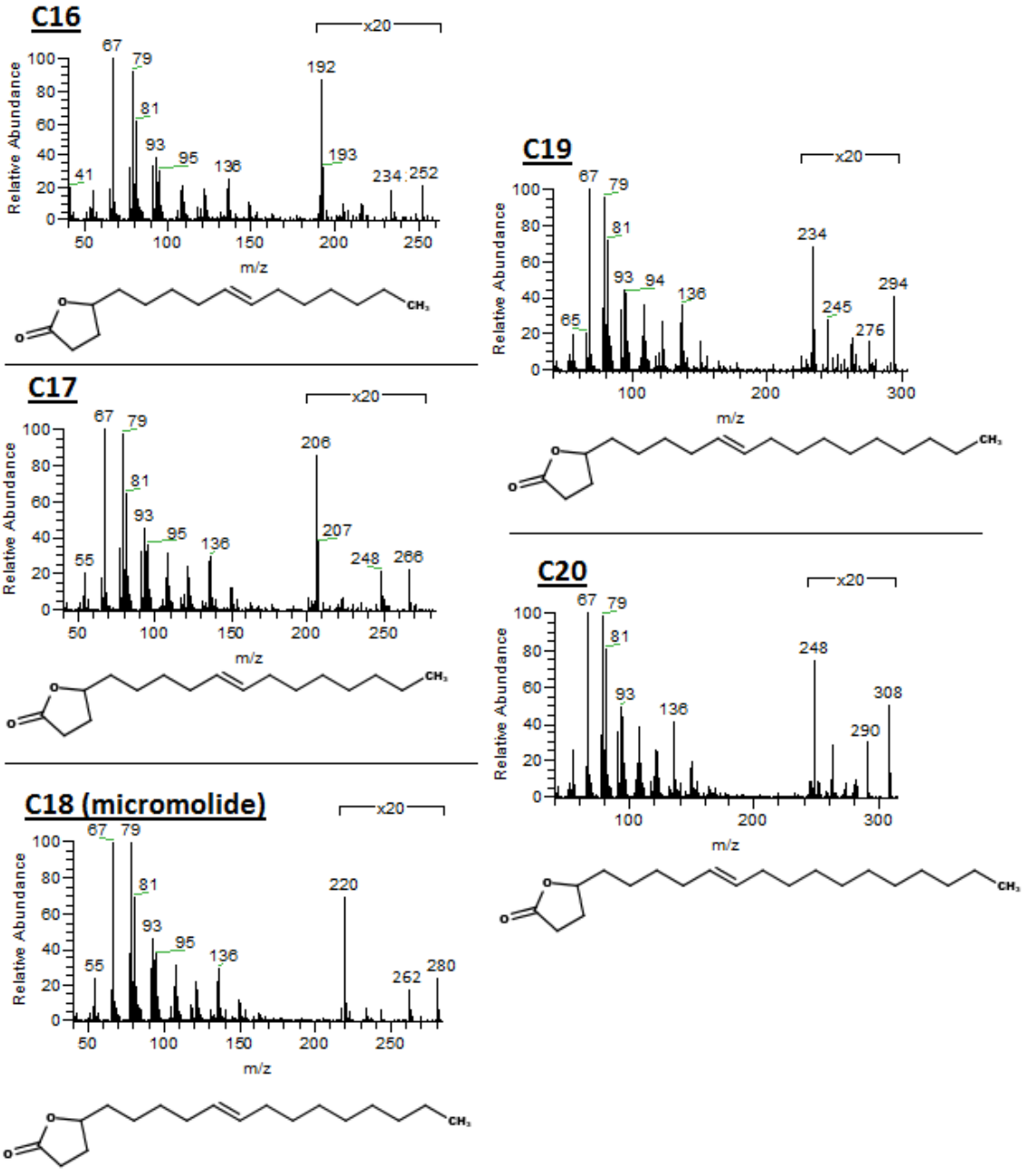
Glossary

DCBP	4,4'-dichlorobenzophenone
DMH	2,6-dimethyl-5-heptenol
EI	Electron Ionisation
GCMS	Gas Chromatography Mass Spectrometry
GLMM	Generalised Linear Mixed Model
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
KI	Kovat's Indices
m/z	Charge to mass ratio
MOR	Mandible Opening Response
MS/MS	Mass Spectrometry/Mass Spectrometry
$NaBH_4$	Sodium borohydride
NaOH	Sodium hydroxide
OFN	Oxygen-free Nitrogen
RT	Retention Time
SIM	Selected Ion Monitoring
TIC	Total Ion Chromatogram
TMS	Trimethylsilyl
VOC	Volatile Organic Compounds

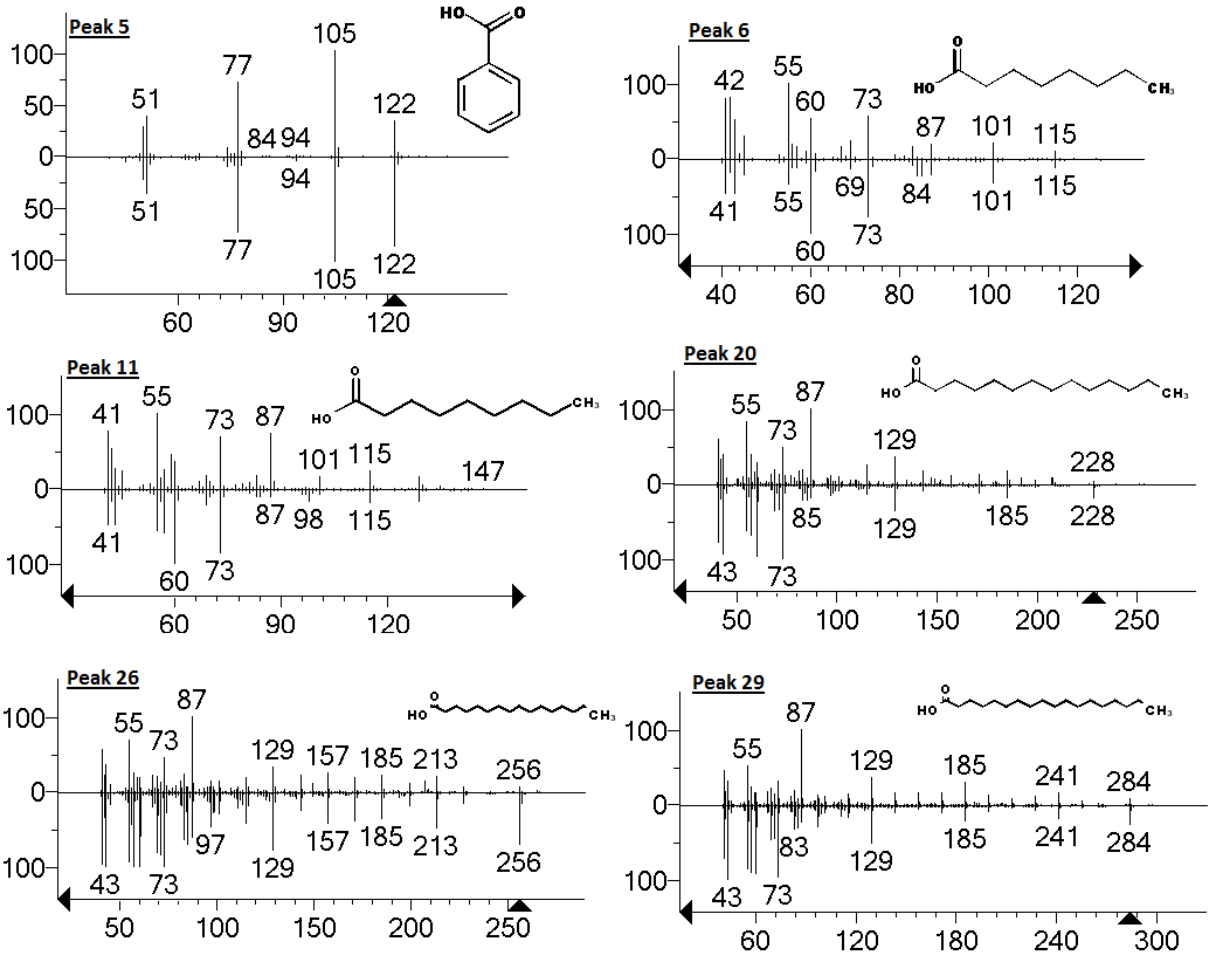
Appendices

Appendix I



The mass spectra and probable chemical structures of putative micromolide homologues detected in Dufour gland extractions detailed in Chapter 2. The corresponding peak numbers from Figure 3 and Table 2 in Chapter 2 are as follows: C16 - #23, C17 - #25, C18 - #26, C19 - #27 and C20 - #28.

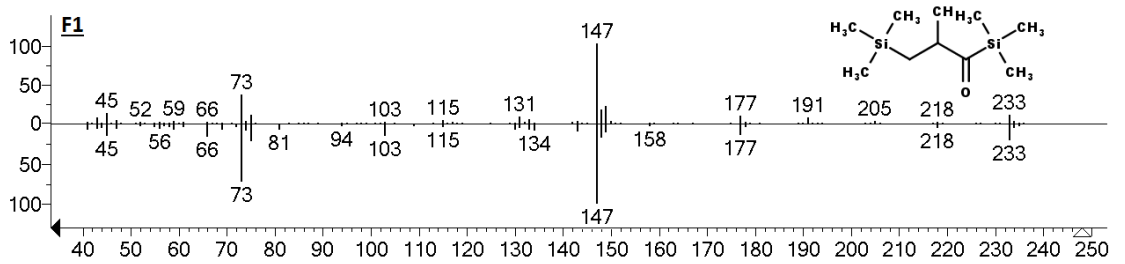
Appendix II

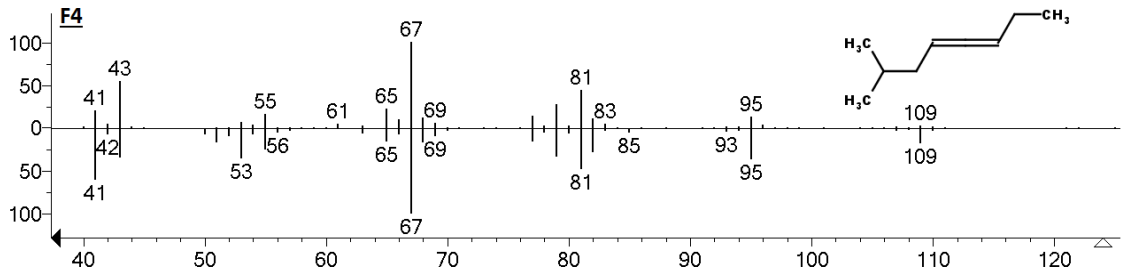


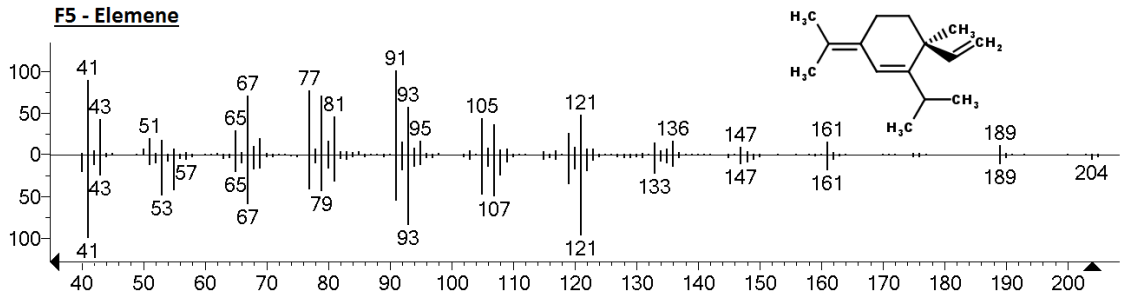
The mass spectra of carboxylic acids detected in poison gland extractions in Chapter 2 match those of authentic standards. In all comparisons, the top spectrum is that of the peak from the poison gland extraction, and the bottom spectrum is that of an authentic standard. The peak numbers correspond with those found in Figure 3 and Table 2 in Chapter 2.

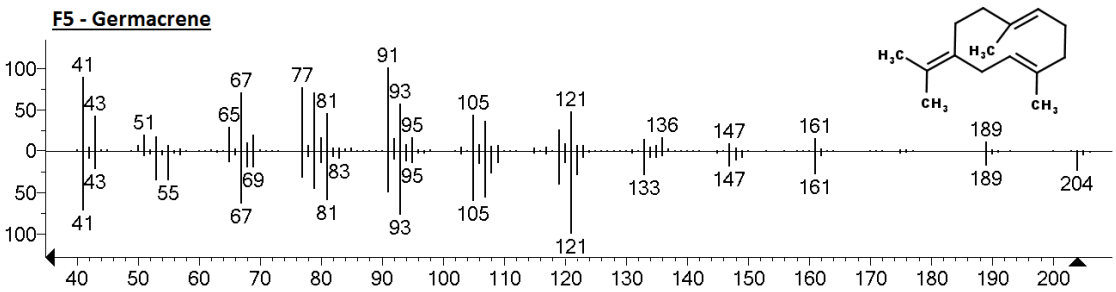
Appendix III

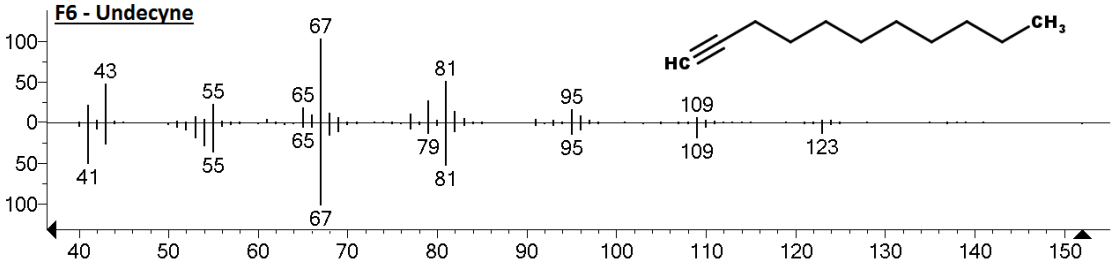
Many of the compounds detected in *Lasius niger* hindguts in Chapter 3 matched very closely with spectra from the NIST/Wiley database. The following figures show comparisons of spectra from hindgut extractions with spectra from the library. Each comparison is labelled with the corresponding peak number from Table 2 in Chapter 3. In all these comparisons, the top spectrum is the spectrum from the hindgut extraction, while the bottom spectrum is from the database. Where multiple database spectra presented a potential match, the name of the compound from the database is also displayed.

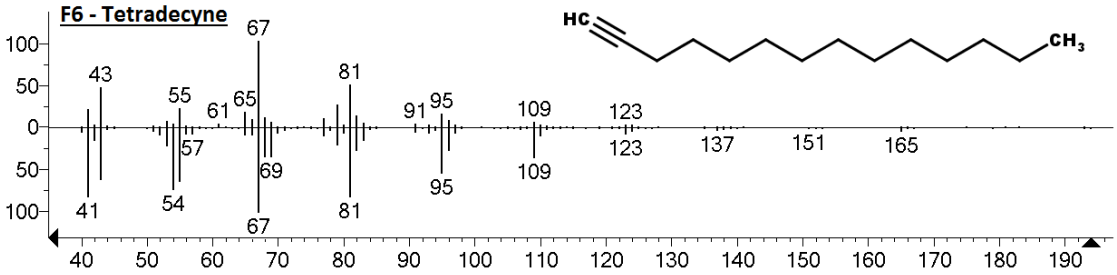


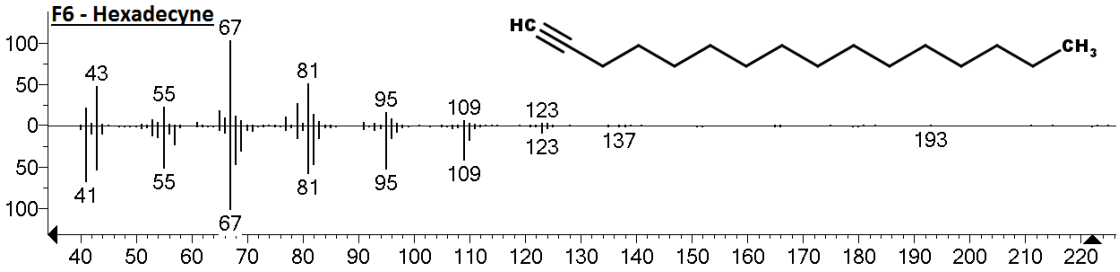


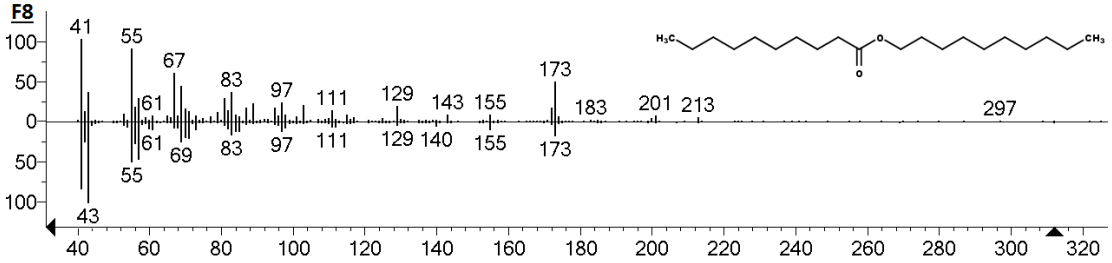


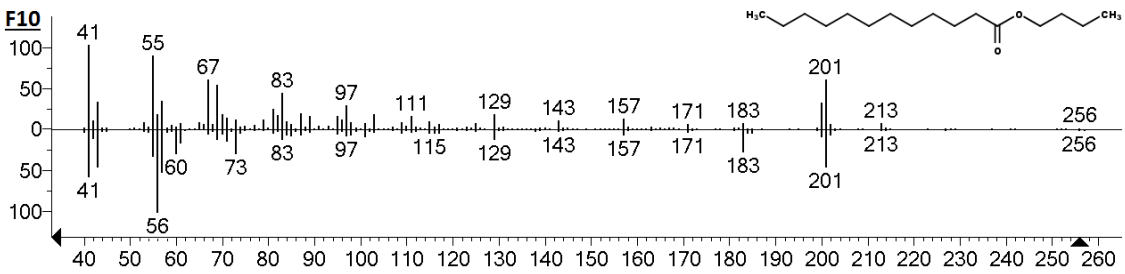

F5 - Germacrene

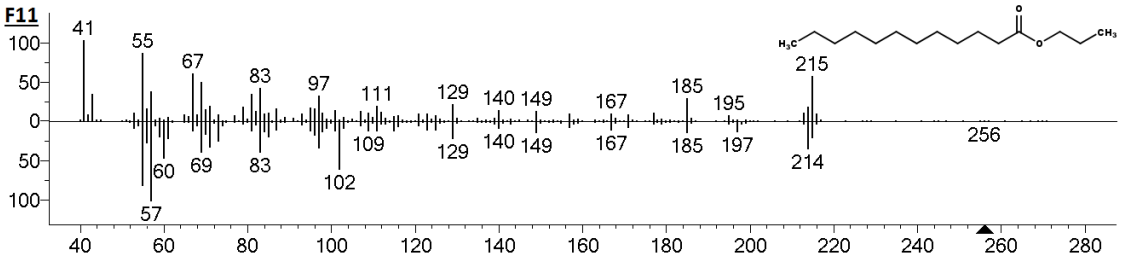

F6 - Undecyne

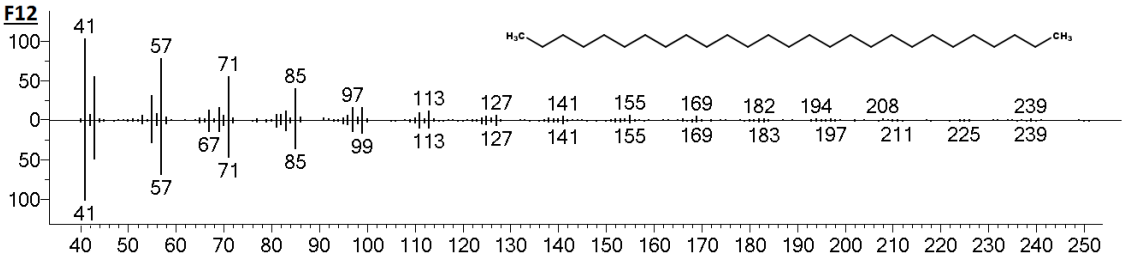

F6 - Tetradecyne

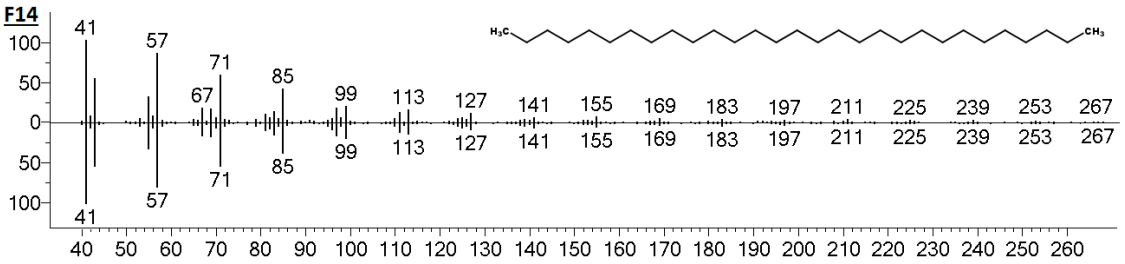

F6 - Hexadecyne

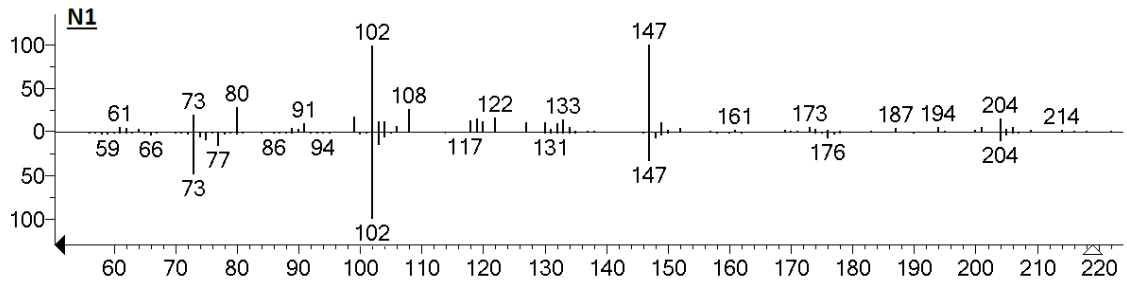

F8

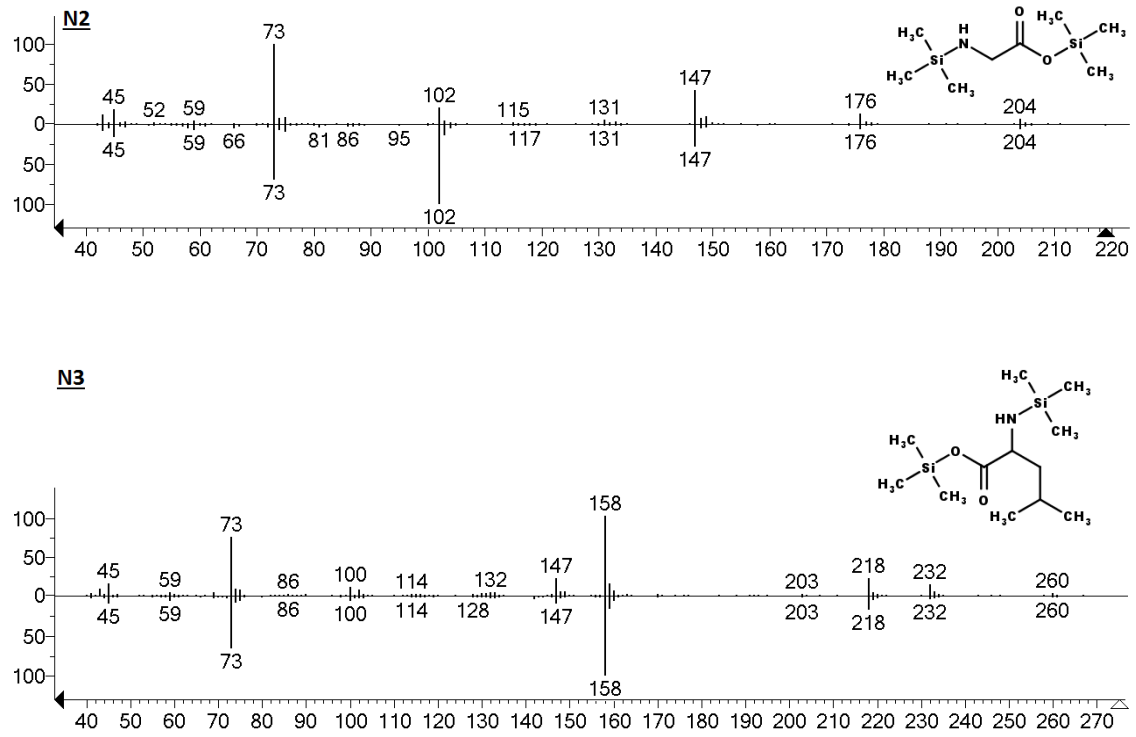

F10


F11

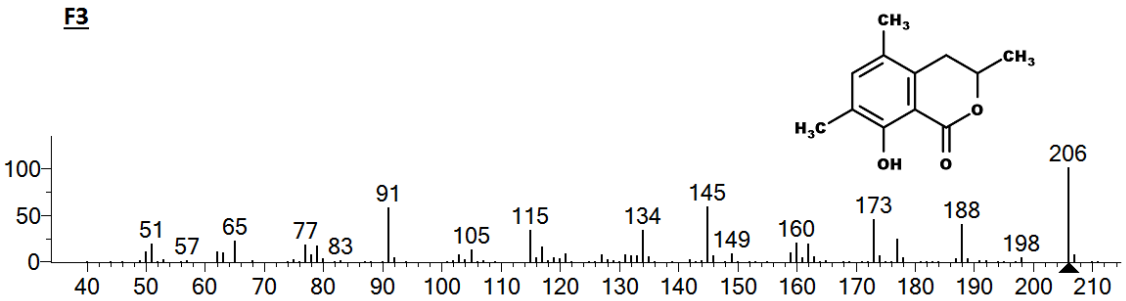

F12


F14


N1

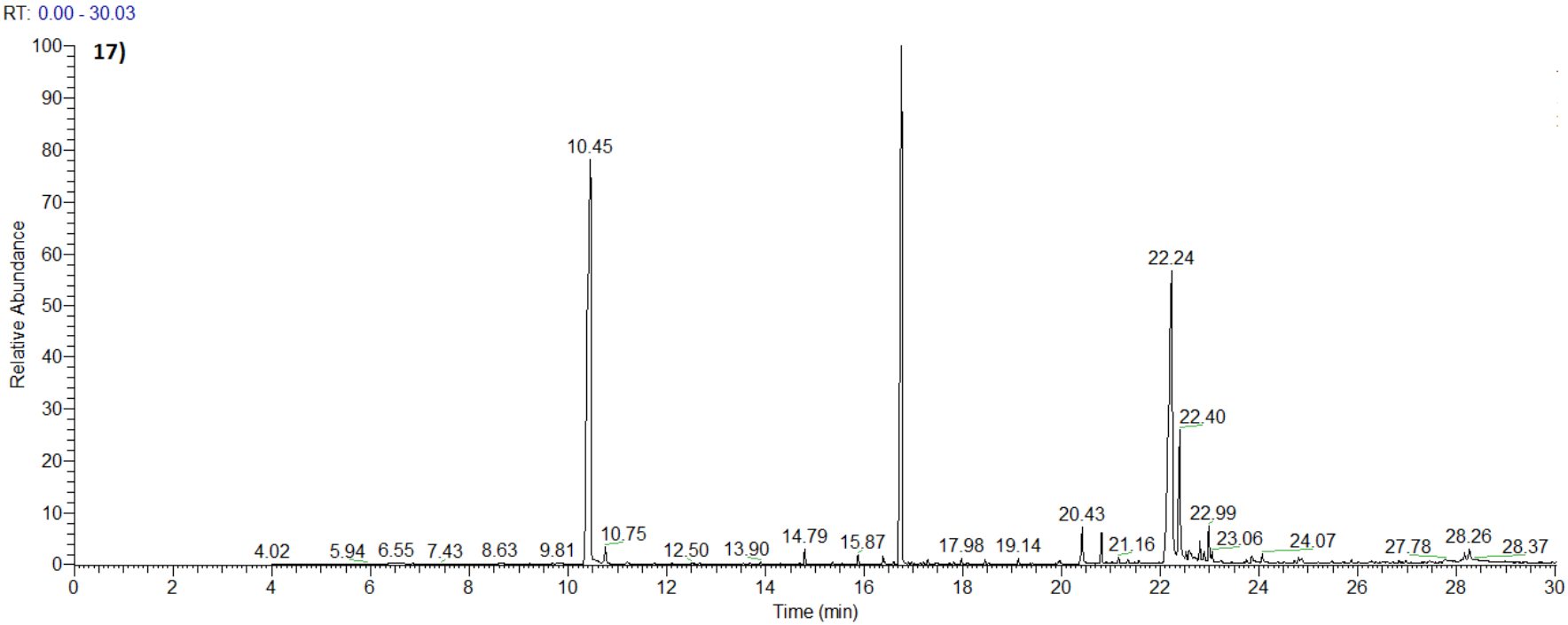


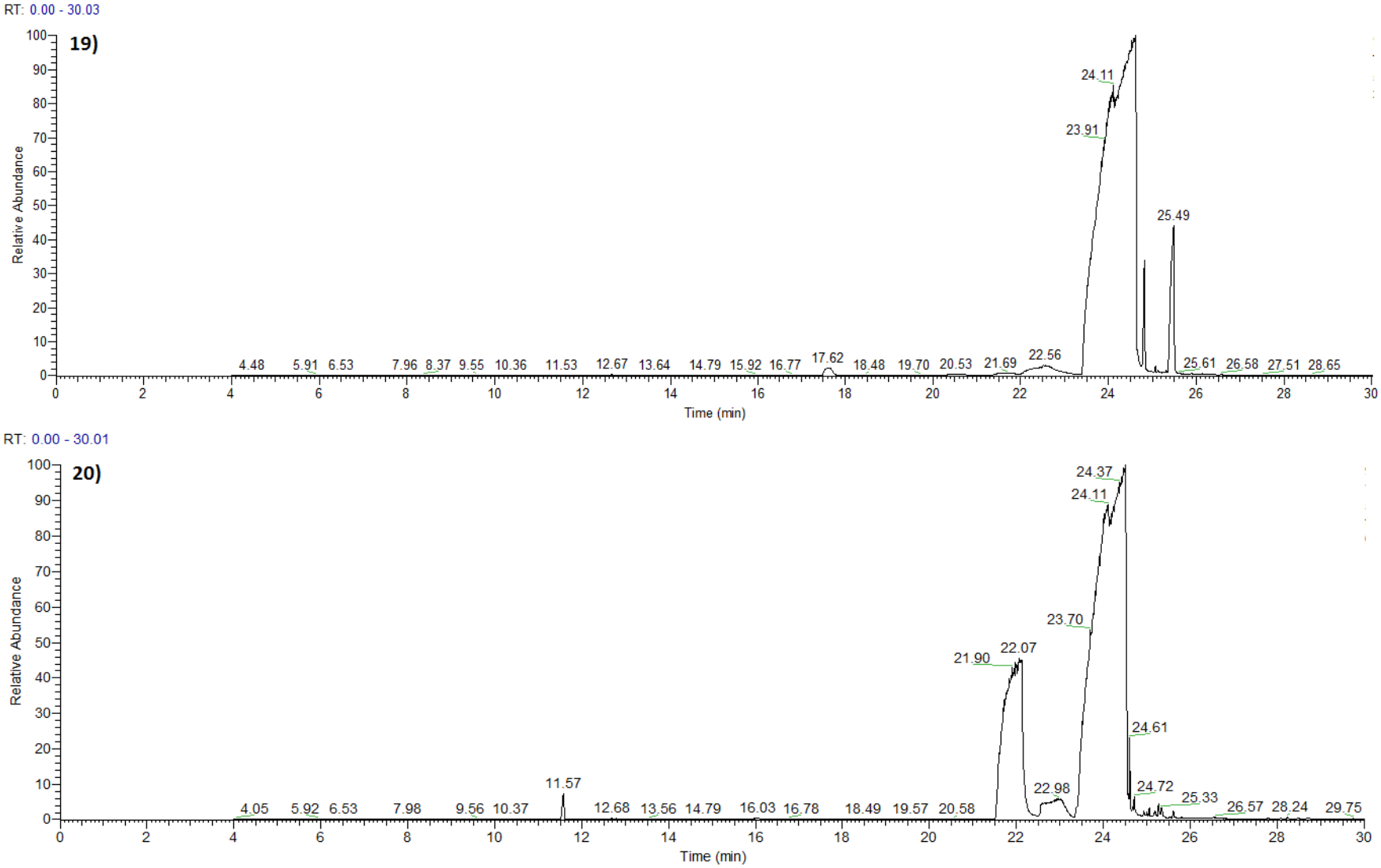
The trail pheromone of *Lasius niger*, 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (F3), was not in the database. It was tentatively identified by comparing its spectrum with the data published by (Bestmann et al., 1992). An image of the mass spectrum was not published, but the peaks and their abundances relative to the largest peak were described as follows: m/z 206 (100), 188 (19), 177 (23), 173 (25), 162 (20), 145 (14), 134 (14). The mass spectrum detected in Chapter 3 in *Lasius niger* hindguts is shown below.



Appendix IV

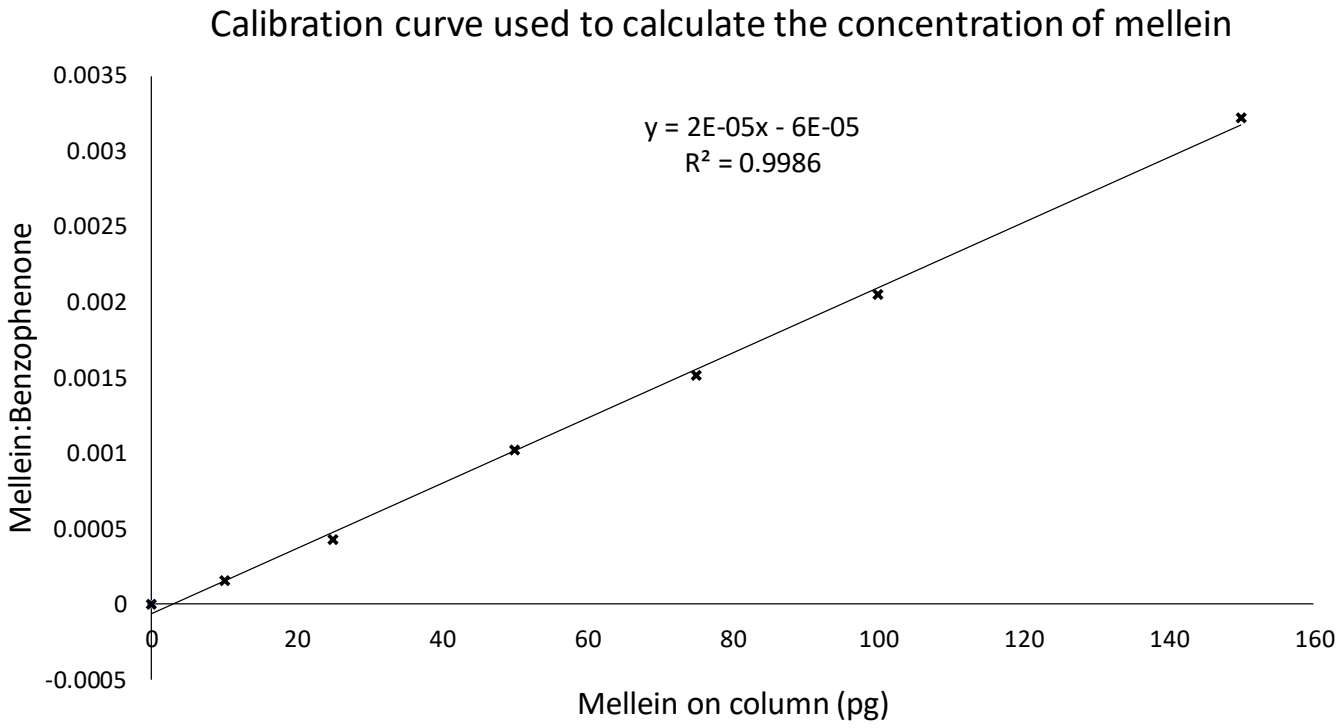
Whole chromatograms for fractions 17, 19 and 20 of the whole body extraction:

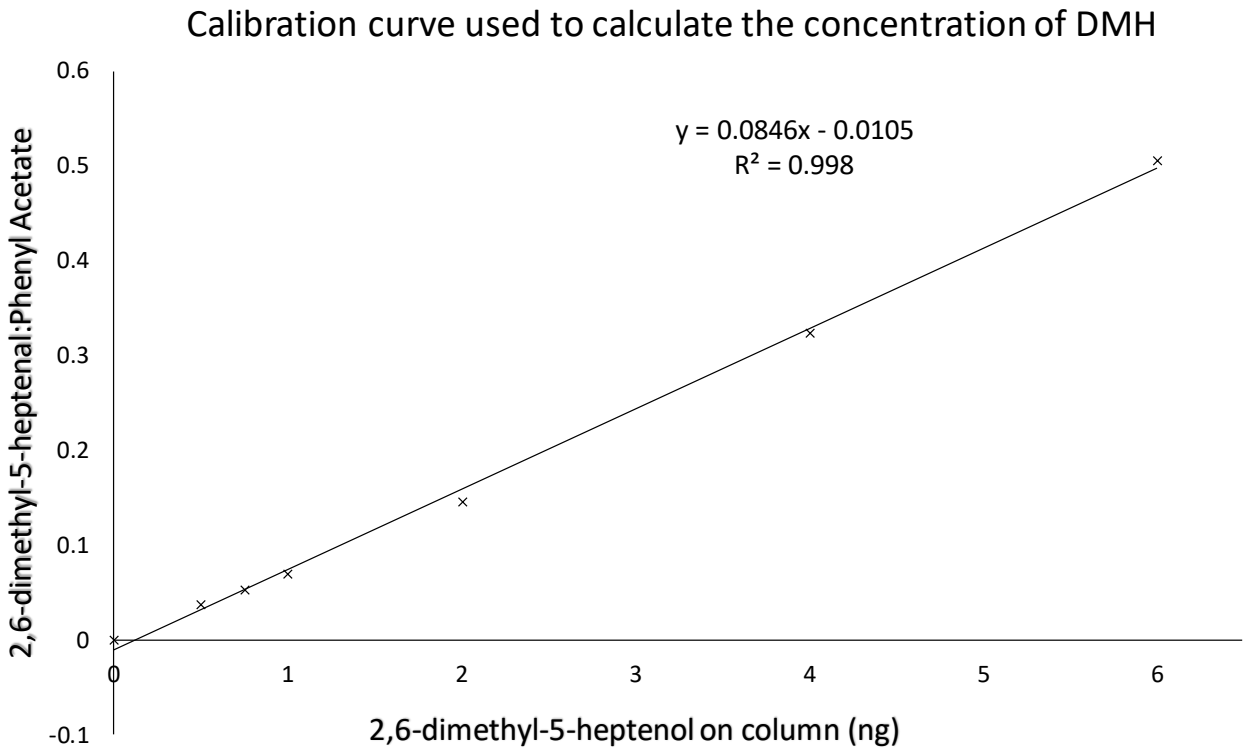



RT: 0.00 - 30.03
19)
Relative Abundance
Time (min)
4.48
5.91
6.53
7.96
8.37
9.55
10.36
11.53
12.67
13.64
14.79
15.92
16.77
17.62
18.48
19.70
20.53
21.69
22.56
23.91
24.11
25.49
25.61
26.58
27.51
28.65
RT: 0.00 - 30.01
20)
Relative Abundance
Time (min)
4.05
5.92
6.53
7.98
9.56
10.37
11.57
12.68
13.56
14.79
16.03
16.78
18.49
19.57
20.58
21.90
22.07
22.98
23.70
24.11
24.37
24.61
24.72
25.33
26.57
28.24
29.75

Appendix V

Calibration curves were used to calculate the concentrations of recruitment pheromones in *Lasius flavus* in Chapter 4. Benzophenone was used as a standard to calculate the concentration of mellein in hindgut extractions and phenyl acetate was used as a standard to calculate the concentration of 2,6-dimethyl-5-heptenal in head extractions from *L. flavus* workers. These calibration curves were generated by analysing a series of seven standard mixtures for each. Each mixture contained the same quantity of a standard compound and different quantities of the target compound. For the mellein curve, all the mixtures contained 1ng/µl of benzophenone, and 0pg/µl, 10pg/µl, 25pg/µl, 50pg/µl, 75pg/µl, 100pg/µl and 150pg/µl of mellein. For the DMH curve, all the mixtures contained 1ng of phenyl acetate, and 0ng/µl, 0.5ng/µl, 0.75ng/µl, 1ng/µl, 2ng/µl, 4ng/µl and 6ng/µl of DMH. 1µl of each mixture was injected onto the GCMS, and the ratio of the abundance of target compound to the standard compound in each mixture was calculated, then plotted against the amount of target compound in the mixtures to create the graphs below. These curves could then be used to calculate the exact amount of DMH or mellein in *Lasius flavus* extractions. A quantity of the relevant standard was added to the extractions so that 1ng of standard was injected onto the GC column, then the ratio of the abundance of the target compound in the extraction to the abundance of the standard was calculated. This ratio was then used, with the relevant calibration curve, to calculate the exact amount of target compound in the extraction.


Calibration curve used to calculate the concentration of mellein
y = 2E-05x - 6E-05
R² = 0.9986
Mellein:Benzophenone
Mellein on column (pg)
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
-0.0005
0
20
40
60
80
100
120
140
160


Calibration curve used to calculate the concentration of DMH
y = 0.0846x - 0.0105
R² = 0.998
2,6-dimethyl-5-heptenal:Phenyl Acetate
2,6-dimethyl-5-heptenol on column (ng)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
1
2
3
4
5
6